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Bromobenzene-Mediated Alteration in Activity and Electrophoretic Pattern of Biliverdin Reductase Variants in Rat Kidney

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SUMMARY

Here we report on the detection of multiple net-charge and molecular mass variants of biliverdin reductase in the rat kidney and describe selective changes in the tissue profile of the variants after bromobenzene treatment (2 mmol/kg, subcutaneously, 24 hr). Using two-dimensional electrophoresis and isoelectric focusing, two major molecular mass species, M_r 30,400 and 30,700, a minor form of M_r 31,400, and five net-charge groups of pI = 6.23, 5.91, 5.77, 5.61, and 5.48 were detected; the net-charge variants with pI = 5.61 and 5.77 were the most abundant forms. The M_r 30,400 form was the main component of two isoelectric focusing bands with pI = 6.23 and 5.91, and the relative amounts of these net-charge variants was severely decreased in the kidneys of bromobenzene-treated rats. The effect of bromobenzene *in vivo* could not be duplicated by *in vitro* experiments involving the direct treatment of purified enzyme with bromobenzene, or incubation of the purified preparation with bromobenzene in the presence of a NADPH-dependent microsomal drug-metabolizing system. Bromobenzene treatment did not alter the immunochemical properties of biliverdin reductase variants, as judged by the similarity of isoelectric focusing patterns of preparations on a Western blot using antibody raised against a rat liver total biliverdin reductase preparation. The treatment, however, caused an alteration in the kinetic properties of the enzyme, and the activity with NADH appeared to be selectively decreased. The possible mechanisms involved in the expression of multiple forms of the reductase and the biological significance of the multiplicity, as well as the change in composition caused by bromobenzene, are discussed.

The conversion of biliverdin, formed in the course of heme oxygenase activity, to bilirubin is catalyzed by biliverdin reductase (reviewed in Ref. 1). The enzyme is present in most mammalian organs, although there are marked differences in tissue levels of activity. The reductase was initially purified to homogeneity from the rat liver in this laboratory (2) and was shown to be unique among eukaryotic enzymes in having two pH optima, i.e., 6.7 and 8.5, utilizing different pyridine nucleotide cofactors at each pH; NADH is used at the lower pH, whereas the reducing capacity of NADPH is exploited at the higher pH value (2, 3). The reductase that was partially purified from the kidney or spleen (4, 5) also displays similar properties. It is noteworthy that a few other enzymes that are known to utilize the reducing potential of two pyridine nucleotides (6, 7) have only one pH optimum.

Recently, we have detected an extensive microheterogeneity of the reductase in the rat liver (8–10). Using two-dimensional electrophoresis, the purified control liver enzyme resolved into five net-charge groups, with pI values of 6.23, 5.91, 5.77, 5.61, and 5.48, and three molecular mass groups, with M_r of 30,400, 30,700, and 31,400 (9). When selected charge groups were examined, differences were noted in their catalytic activity, amino acid composition, and peptide maps (8, 9).

Biliverdin reductase is an —-SH-dependent enzyme and its activity is inhibited in the kidney *in vitro* and *in vivo* by —SH reagents (2, 5) as well as by the substrate, biliverdin (2, 11). Production of biliverdin, in turn, is accelerated in animals treated with various chemicals and stimuli that increase heme oxygenase activity (12–14). Bromobenzene, perhaps the most potent inducer of liver heme oxygenase-1 isozyme (15), is known to be metabolized to reactive metabolites that are capable of interaction with regulatory macromolecules and —SH groups (16–18). It is noteworthy that, in the preparation purified from the liver of bromobenzene-treated rats and examined by two-dimensional electrophoresis system, a marked alteration in the tissue profile of biliverdin reductase variants is observed; in particular, a major depression in the M_r 30,400 form of the reductase is detected (10).

The present study was undertaken to examine whether multiplicity of the reductase extends to the kidney and, if so, whether bromobenzene treatment alters the electrophoretic profile of variant(s) in this organ. The kidney was selected for

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ABBREVIATIONS: DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing.

this study because this organ is a target for bromobenzene metabolites that are apparently formed in the liver and carried by blood to the organ (19).

Experimental Procedures

Materials and treatment of animals. Chromatographic media, ampholytes, and biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). NADP-agarose, Type 3, and Ultrogel AcA54 were purchased from Pharmacia-LKB and IBF Biotechnics (Savage, MA), respectively. Biliverdin IXα was obtained from Porphyrin Products (Logan, UT). All chemicals were of the highest purity commercially available. Goat anti-rabbit IgG conjugated with horseradish peroxidase was purchased from Organon Teknika Corporation (West Chester, PA). Nitrocellulose filters with a 0.2-μm pore were obtained from Schleicher and Schuell. Male Sprague-Dawley rats (200–250 g) were purchased from Harlan Industries (Madison, WI) and male New Zealand rabbits (3–4 kg) were purchased from Hazelton Research Animals (Denver, PA).

Purification methods. Biliverdin reductase was purified from the kidneys of control rats or animals that were treated subcutaneously with 2 mmol/kg bromobenzene and killed 24 hr later. The purification protocol consisted of a modification of the method previously described (2). For a typical purification, organs from 150–200 rats were pooled. Kidneys were homogenized in 5 volumes of a 20.00 mM potassium phosphate buffer, pH 7.4, that contained 1.0 mM EDTA and 135.0 mM KCl. The cytosol fraction, which was prepared by centrifugation at 150,000 × *g* for 1 hr, was diluted with 1/19 volume of a 1.0 M citrate buffer, pH 5.4, which contained 2.0 mM DTT before ammonium sulfate fractionation. Group-specific chromatography with a NADP-agarose column was performed as detailed before (2), except that the enzyme was eluted from the column with equilibration buffer, which contained 50.0 mM glycine and 100.0 mM triethanolamine and was adjusted to pH 9.0. The active fractions were pooled, concentrated, and subjected to gel filtration using an Ultrogel AcA54 chromatographic column. The mobile phase contained 0.1 M Tris, 0.1 mM EDTA, 10.0% (v/v) glycerol, and 0.2 mM DTT and was adjusted to pH 7.5 with HCl. The pooled active fractions were desalted on a Sephadex G-25 column with a mobile phase that contained 10.0 mM Tris, 0.1 mM EDTA, 20.0% (v/v) glycerol, and 0.2 mM DTT and were adjusted to pH 7.5. The active fractions were pooled, concentrated, and stored at −80°. The purified biliverdin reductase preparation was judged to be homogeneous by SDS-PAGE. A similar procedure was used for purification of the reductase from the liver. This preparation was used for production of antibody in the rabbit, as described before (20).

In vitro treatments with bromobenzene. Two regimen of bromobenzene treatment were used. In one experiment, the reductase purified from control kidneys was incubated at 37° for 15 min with 2.0 mM bromobenzene and was used for polyacrylamide gel IEF analysis. In another experiment, the liver microsomal fraction was prepared from control rats. Purified control kidney biliverdin reductase was incubated for 15 min at 37° with bromobenzene (2.2 mM, final concentration) in an assay system (440 μl) containing the liver microsomes (0.2 mg of protein) and NADPH (0.5 mM). Thereafter, the reaction mixture was centrifuged 150,000 × *g* and the supernatant fraction, containing kidney biliverdin reductase, was retrieved, concentrated, and used for IEF analysis.

Electrophoretic and immunochemical methods. SDS-PAGE was performed by the method of Laemmli (21). The separating gels contained 10.0% *T* and 2.67% *C* and the stacking gels contained 3.0% *T* and 2.67% *C*, where *T* was the total acrylamide concentration (w/v) and *C* was the ratio of bisacrylamide to total acrylamide (w/w). β-Galactosidase (M_r 116,000), phosphorylase *b* (M_r 97,400), serum albumin (M_r 66,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 29,000), and trypsinogen (M_r 24,000) were used as molecular weight markers.

The method used for IEF was based upon that described by Righetti and Drysdale (22). The separating gels contained 4.0 *T* and 5.4% *C*. pH 5–8 range and pH 3.5–10 range carrier ampholytes were used and their concentrations were adjusted to 1.8% (w/v) and 0.2% (w/v), respectively. Electrofocusing was performed at 400 V for 17 hr and then continued for 1.5 hr at 800 V. Amyloglucosidase from *Aspergillus oryzae* (pI 3.55), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.13), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.57), and horse heart myoglobin (pI 6.76 and 7.16) were used as pI markers.

Two-dimensional PAGE was performed essentially by the method originally described by O'Farrell (23). The conditions that were used for both the first dimension IEF tube gel separation and the second dimension SDS-PAGE separation were similar to those that were described above for the single-dimension separations.

Single-dimension IEF tube gels were stained with Coomassie brilliant blue R250 (22). The positive-image silver staining method of Wray *et al.* (24) was used for the staining of slab gel IEF and two-dimensional PAGE gels. Laser densitometry and automatic integration were performed with an LKB Ultroscan XL densitometer.

Western immunoblotting subsequent to IEF was performed by the method of Towbin *et al.* (25). Primary and secondary antibody treatments followed by peroxidase activity staining with 4-chloro-1-naphthol were performed as described before (26).

Assay procedures. Protein was measured by the method of Lowry *et al.* (27). Bovine serum albumin was used as the protein standard. Routine measurements of purified biliverdin reductase activity were performed as described before (2). The reaction was initiated by the addition of 60.0 μl of either 1.0 mM NADH or 0.1 mM NADPH, which were prepared in 0.1 mM EDTA, pH 7.4, to the test reaction mixture. The reaction was monitored for 30 to 60 sec at 25°.

Results

The IEF pattern of a purified preparation of control rat kidney biliverdin reductase is shown in Fig. 1. The slab gel system, which contained a mixture of pH 5–8 ampholine and pH 3.5–10 ampholine, resolved five major electrophoretic zones of dissimilar intensities, when visualized by silver staining,

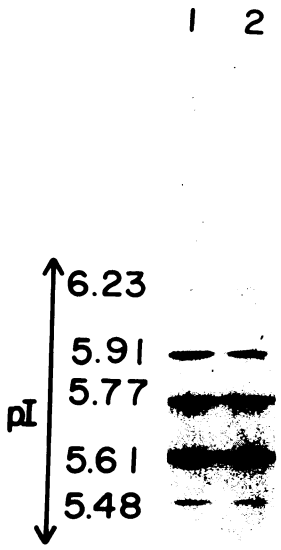

Fig. 1. Slab gel IEF of control kidney biliverdin reductase. Biliverdin reductase was purified from control male Sprague-Dawley rat kidneys. Three micrograms of purified enzyme preparation were separated by IEF, as described in Experimental Procedures. IEF bands were visualized by positive-image silver staining. *Lane 1* and *2* contained the same preparation.

within the preparation. The electrophoretic zones, which were interpreted as net-charge variants of biliverdin reductase and were designated as IEF-1 to IEF-5, were associated with the following pI values: IEF-1 = 6.23, IEF-2 = 5.91, IEF-3 = 5.77, IEF-4 = 5.61, and IEF-5 = 5.48. The possibility that the kidney biliverdin reductase may display heterogeneity with respect to molecular mass was examined using two-dimensional electrophoresis (Fig. 2). The first-dimension IEF tube gel was loaded with 3 μg of purified biliverdin reductase protein. As shown in Fig. 2, the enzyme preparation separated once again into five net-charge groups of dissimilar pI values. Furthermore, in the second dimension, biliverdin reductase separated clearly into two major M_r groups of 30,400 and 30,700; a minute amount of a variant with M_r 31,400 in IEF-4 (pI = 5.61) was also detected.

The effect of bromobenzene treatment (2 mmol/kg, subcutaneously, 24 hr) on kidney biliverdin reductase IEF variants was examined using a purified preparation obtained from the treated rats (Fig. 3, *lane 2*) and compared with a control preparation (Fig. 3, *lane 1*). Both lanes contained the same amount of protein (6 μg), and the protein bands were visualized using Coomassie blue. As noted, the IEF patterns of the two preparations differed; in the preparation obtained from treated rats, the intensity of IEF-1 was markedly diminished and that of IEF-2 was also visibly decreased. The relative amounts of each variant of biliverdin reductase in the two preparations were quantitated by laser densitometry. The results are shown in Table 1. In both preparations, the IEF-4 (pI = 5.61) and IEF-3 (pI = 5.77) variants were the most abundant forms; these were followed by IEF-2 form (pI = 5.91). In the bromobenzene-treated rats, however, the relative amounts of IEF-1 and IEF-2 were decreased when compared with the control; IEF-1 measured about 3% of the total area and IEF-2 amounted to approximately 10% of the total and, in the control preparation, the corresponding values were 11% and 18%, respectively. It should be noted that the IEF values and the effect of bromobenzene on IEF-1 and IEF-2 were highly reproducible in preparations purified from different groups of rats. In order to gain some understanding of the cellular and chemical basis for change caused by bromobenzene treatment in the IEF pattern of biliverdin reductase variants, the following experiments were carried out. In one experiment, the direct effect of bromobenzene on biliverdin reductase was examined by treating *in vitro* a control preparation of purified kidney reductase with 2.0 mM bromobenzene (15 min at 37°) before IEF analysis; the pattern of separation and the pI values for the net-charge variants were essentially identical to those shown in Fig. 1 for the control preparation (data not shown). To further examine the possibility of a direct interaction of bromobenzene metabolite(s) with the enzyme, the following experimental protocol, which is based on that recently described for glutathione *S*-transferases (28), was carried out. Purified biliverdin reductase from the control

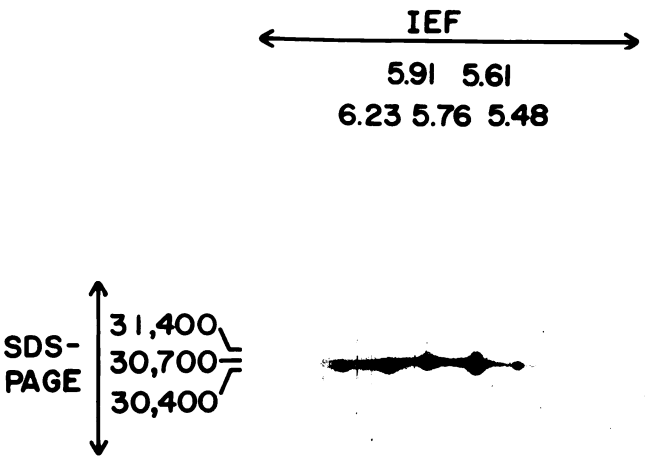

Fig. 2. Two-dimensional electrophoresis of purified rat kidney biliverdin reductase. In the first dimension, 3 μg of biliverdin reductase purified from control rat kidneys were separated by tube gel IEF. Subsequently SDS-PAGE was used for the second dimension. Experimental details are provided in the text. Molecular mass and charge variants were visualized by positive-image silver staining.

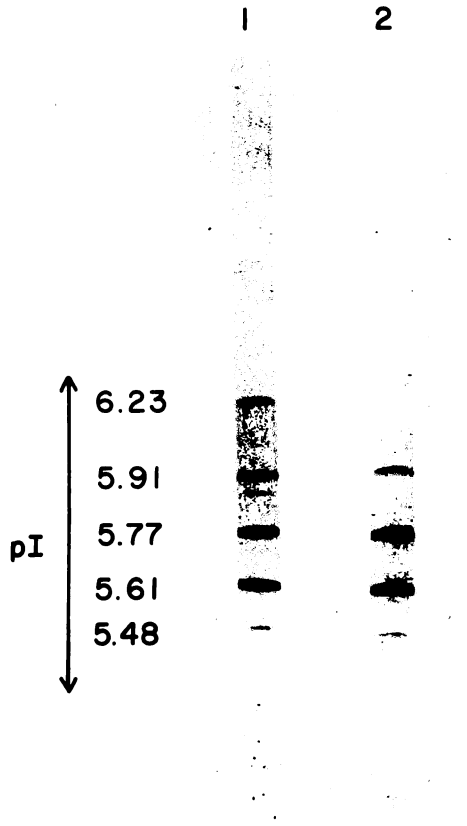

Fig. 3. Tube gel IEF of purified biliverdin reductase obtained from control and *in vivo* bromobenzene-treated rat kidneys. Biliverdin reductase was purified from control and bromobenzene-treated (2 mmol/kg, subcutaneously) rats. Six micrograms of protein were used for IEF analysis. The net-charge variants were visualized by Coomassie blue staining. *Lane 1*, untreated; *lane 2*, bromobenzene-treated.

TABLE 1

Relative densitometric area of biliverdin reductase net-charge variants in the kidneys of control and bromobenzene-treated rats

Purified enzyme preparations obtained from the kidneys of control and bromobenzene-treated rats (2 mmol/kg, subcutaneously) were subjected to tube gel IEF and stained with Coomassie blue, as described in Experimental Procedures. The relative area was measured by laser densitometry of the gels.

Designation	pI	Relative area	
		Control	Bromobenzene-treated
		% *of total*	
IEF-1	6.23	11.5	2.8
IEF-2	5.91	18.5	10.3
IEF-3	5.77	29.1	40.9
IEF-4	5.61	38.4	41.1
IEF-5	5.48	2.6	4.4

rat kidneys was incubated (15 min, 37°) with bromobenzene (2.2 mM) in the presence of a liver microsomal drug-metabolizing system. The reductase was subsequently retrieved and subjected to slab gel IEF. Again, no difference between the IEF pattern of the treated and untreated enzyme preparations could be detected (data not shown). Therefore, it appears unlikely that a direct interaction of the enzyme with bromobenzene itself or its metabolite(s), which could have been formed in the course of biotransformation of the compound under the conditions of incubation used in the experiment, was responsible for the observed changes in the IEF pattern of reductase purified from the liver of bromobenzene-treated rats.

The following experiment was conducted to examine whether bromobenzene treatment *in vivo* alters immunochemical properties of the variants. The Western immunoblotting technique using antibody raised against total biliverdin reductase purified from control rat liver was utilized. Fig. 4 shows the Western immunoblot of slab gel IEF separation of the reductase obtained from the kidneys of bromobenzene-treated rats (Fig. 4, *lane 1*) and control animals (Fig. 4, *lane 2*). Experimental conditions were similar to those described for Fig. 1, except that 6 μg of reductase protein were used. According to the findings, bromobenzene treatment did not appear to change the immunochemical properties of the variants. As noted, the number and position of components on the immunoblot in the control preparation closely resembled those seen in Fig. 1 and, as above (Fig. 3), in the preparation obtained from bromobenzene-treated rats, the relative amounts of IEF-1 and IEF-2 were clearly decreased.

In order to examine whether changes caused by bromobenzene treatment in biliverdin reductase variants were reflected in enzyme activity, the specific activity of the reductase purified from control and treated rats using NADH or NADPH as cofactors was measured. Data are shown in Table 2. As noted, the ratio of activity at the two pH optima was dissimilar for the enzyme preparations purified from the control and the treated rats. In the control preparation, the ratio approached unity. However, in the preparation obtained from the bromobenzene-treated rats, the rate of activity with pyridine nucleotide cofactors was differentially affected, with the NADH-dependent activity being severely decreased, leading to a major deviation from unity of the activity ratio.

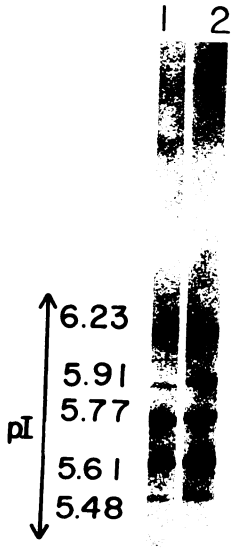

Fig. 4. Western immunoblot of slab gel IEF separation of purified biliverdin reductase from kidneys of control or *in vivo* bromobenzene-treated rats. The slab gel IEF separation of biliverdin reductase preparations was carried out as described in Experimental Procedures, using 6 μg of enzyme protein. This was followed by electroblotting onto a nitrocellulose sheet and immunochemical staining using antibody raised against total liver biliverdin reductase. Details are provided in the text. *Lane 1*, *in vivo* bromobenzene-treated; *lane 2*, control.

Discussion

The present study describes detection of multiple forms of biliverdin reductase in the kidney. This phenomenon also has been noted in the liver and the spleen (8–10). However, there are major differences in the composition of the variants in different organs. For instance, a variant with pI = 5.75 that is present in the liver is apparently absent in the kidney. Moreover, although forms of M_r 30,700 and 30,400 were the most abundant forms in the liver, a substantial amount of M_r 31,400 form was also present in this organ (9). In the kidney, however, the latter form was minimally detectable (Fig. 2). In the liver, we have noted that IEF-4 was the major variant, amounting to about 40% of the total, followed by IEF-3 (pI = 5.77), which constituted about 25% of the total (9). In the kidney, these variants also constituted the most prevalent forms (Table 1). On the other hand, in the spleen, a variant with pI = 6.23 was nearly absent and pI = 5.91 was present in a minute amount (10). At the present time, the cellular basis for the extensive microheterogeneity of biliverdin reductase is not evident; also the molecular basis for the seemingly selective decrease in cellular levels of IEF-1 and IEF-2 variants by bromobenzene is not clear. Apparently the heterogeneity of biliverdin reductase extends to other species and, recently, in the ox kidney, two charge isomers of the reductase (pI 5.4 and pI 5.2) have been detected (28).

Various hypotheses, however, can be offered in explanation of the observations. The occurrence of a gene family for the reductase could generate polypeptides that are very similar but have unequal molecular masses, as is the case with actin gene family (29). The minimum amino acid divergence is exemplified by α-skeletal and α-cardiac actins, which show only four amino acid changes out of 375 residues (30). Our recent finding with amino acid composition analysis of two of the variants separated by nondenaturing electrophoresis of liver biliverdin reductase (8) is consistent with the existence of more than one gene. However, other possibilities such as alternative mRNA

TABLE 2

Effect of bromobenzene treatment on NADH- or NADPH-dependent activity of purified kidney biliverdin reductase

Male Sprague-Dawley rats were treated with bromobenzene (2 mmol/kg, subcutaneously). The animals were killed 24 hr later and enzyme was purified from the kidneys. Control enzyme was prepared from untreated rats. The activity of the preparations was measured using the indicated pH values and cofactors and is expressed as the nmol of bilirubin formed. Experimental protocol are described in detail in the text.

Treatment	Specific activity		Activity ratio, NADH/NADPH
	NADH (pH 6.7)	NADPH (pH 8.5)	
	nmol/mg/min		
Control	2213	2405	0.92
Treated	1581	2433	0.65

splicing of a single gene product (31) as well as various post-translational processing of the product of a single gene could also lead to the heterogeneity of charge and molecular mass of the variants. As noted in Figs. 3 and 4, IEF-1 and IEF-2 were the net-charge variants that were most dramatically affected in the kidneys of *in vivo* bromobenzene-treated rats. Because, as shown in Fig. 2, IEF-1 and IEF-2 are the species that consist exclusively of the M_r 30,400 form of the reductase, it is reasonable to suggest that bromobenzene treatment mainly suppresses the cellular content of this form of the enzyme. It follows that, should variants represent multiple gene products, it would appear that bromobenzene treatment *in vivo* selectively affects the expression of one or more gene(s); on the other hand, should the variants represent the posttranslational modifications of one gene loci, then it would appear that bromobenzene treatment selectively interferes with posttranslational modifications. Based on present findings with *in vitro* experiments, as well as previous *in vitro* studies with liver enzyme using nondenaturing electrophoretic conditions (8), it seems unlikely that bromobenzene and/or its metabolites directly modify the fully processed enzyme protein. In this respect, biliverdin reductase differs from glutathione-*S*-transferases, whose isozymes have been shown to be selectively arylated by bromobenzene metabolites (18). In any event, because bromobenzene is metabolized by cytochrome P-450-dependent mixed-function oxidase to active metabolites that can bind to cellular macromolecules (16–18), there is a distinct possibility that interaction of metabolite(s) with critical regulatory macromolecules is responsible for the observed changes in the pattern of production of biliverdin reductase variants.

The physiological significance for the existence of multiple forms of the reductase may be related to the previously demonstrated difference in their kinetic properties (8, 9) as well as the potential differences in their substrate specificity or kinetics of inhibition by the bile pigments and metalloporphyrins, i.e., Zn-protoporhyrin (2, 3, 11). Presently, we are further investigating the kinetic properties of biliverdin reductase variants as well as the molecular basis for heterogeneity of the enzyme.

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