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Measurement of Membrane-Bound Human Heme Oxygenase-1 Activity Using a Chemically Defined Assay System

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ABRREVIATIONS USED:

Heme oxygenase (HO); heme oxygenase-1, full-length (HO-1); nicotinamide adenine dinucleotide phosphate, reduced form (NADPH); NADPH-cytochrome P450 reductase (CPR); 30-kDa soluble hHO-1 (sHO-1); human biliverdin reductase (hBVR); reactive oxygen species (ROS); superoxide dismutase (SOD); hydrogen peroxide (H_2O_2); carbon monoxide (CO); ferrous iron (Fe^{2+}); dilauroylphosphatidylcholine (DLPC); ethylenediaminetetraacetic acid (EDTA); bovine serum albumin (BSA); reconstituted systems (RCS); endoplasmic reticulum (ER); extinction coefficient (ε)

ABSTRACT

Heme oxygenase (HO) catalyzes heme degradation in a reaction requiring NADPH-cytochrome P450 reductase (CPR). Although most studies with HO used a soluble 30-kDa form, lacking the C-terminal membrane-binding region, recent reports demonstrate that the catalytic behavior of this enzyme is very different if this domain is retained; the overall activity being elevated 5-fold, and the K_m for CPR decreased approximately 50-fold. The goal of these studies was to accurately measure HO activity using a coupled assay containing purified biliverdin reductase (BVR). This allows measurement of bilirubin formation after incorporation of full-length CPR and heme oxygenase-1 (HO-1) into a membrane environment. When rat liver cytosol was used as the source of partially purified BVR, the reaction remained linear for 2-3 minutes; however, the reaction was only linear for 10-30 sec when an equivalent amount of purified, human BVR (hBVR) was used. This lack of linearity was not observed with soluble HO-1. Optimal formation of bilirubin was achieved with concentrations of bovine serum albumin (0.25 mg/ml) and hBVR (0.025-0.05 μM), but neither supplement increased the time that the reaction remained linear. Various concentrations of superoxide dismutase had no effect on the reaction; however, when catalase was included, the reactions were linear for at least 4-5 minutes, even at high CPR levels. These results not only demonstrate that HO-1-generated hydrogen peroxide leads to a decrease in HO-1 activity, but also provide for a chemically defined system to be used to examine the function of full-length HO-1 in a membrane environment.

INTRODUCTION

Heme oxygenase-1 (HO-1) is a membrane-bound enzyme that initiates the oxidative cleavage of heme to carbon monoxide (CO), Fe^{2+}, and biliverdin, which is further degraded to bilirubin by the cytosolic enzyme, biliverdin reductase (Tenhunen *et al.*, 1969). HO-1 is the inducible isoform of the enzyme responsible for maintaining heme homeostasis in an array of species (Maines *et al.*, 1986), including humans, in which spleen, liver, and kidney are the main sites of expression (Tenhunen *et al.*, 1968;Martasek *et al.*, 1988). This regulation is essential because free heme is highly toxic (Alam, 2002), but more importantly, the metabolites of heme degradation mediate a variety of vital physiological processes. Bilirubin has been well documented as an initial defense mechanism against cellular oxidative stress and tissue inflammation in response to multiple stimuli (Maines, 1992;Stocker *et al.*, 1987;Dore *et al.*, 1999;Willis *et al.*, 1996;Lee and Chau, 2002;Keyse and Tyrrell, 1989). Regulation of heme metabolism ensures efficient sequestration and recycling of free iron, the second HO-1 metabolite, for the synthesis of other critical hemoproteins (Maines, 1997). The third metabolite, CO is known to act as a potent vasodilator and anti-inflammatory molecule via cellular signaling cascades akin to nitric oxide (Otterbein *et al.*, 2000;Verma *et al.*, 1993;Stevens and Wang, 1993;Ryter *et al.*, 2002).

HO-1 activity has been measured using several techniques. Although biliverdin is a primary metabolite of the HO-1/heme reaction, it is rarely used as an indicator due to poor spectral properties, having an extinction coefficient (ε) of only ~ 8 – 10 mM^{-1} cm^{-1} (Kutty and Maines, 1981). Thus, one of the most common HO-1 activity assays relies on the reduction of biliverdin to bilirubin, as originally described by Tenhunen and

colleagues (Tenhunen *et al.*, 1968;Tenhunen *et al.*, 1969). In this method, bilirubin formation is monitored spectrophotometrically by the increase in absorbance at 468 nm (ε_{468} = 43.5 mM^{-1} cm^{-1}), which is approximately five-fold higher than that of biliverdin. Variations of this assay can be found throughout the literature, but all rely on the formation of bilirubin as the marker of HO-1 activity. Maines and Kappas measured HO-1 activity by monitoring bilirubin formation using the difference in absorbance at 464-530 nm ($\varepsilon_{464-530}$ = 40 mM^{-1} cm^{-1}) (Maines and Kappas, 1974;Maines, 1996). Others have used CO production as a method to assay HO-1 activity (Vreman and Stevenson, 1988), but it is less commonly used due to its complexity and the required subsequent product analysis.

Bilirubin formation requires a contribution from HO-1, CPR, and BVR, which are thought to form a multi-enzyme complex (Tenhunen *et al.*, 1968;Yoshinaga *et al.*, 1982b). HO-1 and CPR are bound to the ER membrane, as HO-1 requires electrons shuttled through CPR (Schacter *et al.*, 1972), whereas BVR is a cytosolic protein. NADPH supplies electrons to CPR and they are transported via the flavin groups, FAD and FMN, to HO-1, initiating heme catalysis (**Figure 1**). Heme is oxidatively metabolized to biliverdin, which dissociates from the HO-1 and is rapidly converted to the hydrophobic molecule, bilirubin, in an NADPH-dependent reaction catalyzed by cytosolic BVR. Liver cytosol has been used as a source of BVR (Tenhunen *et al.*, 1970). However, the availability of purified rat (Kutty and Maines, 1981) and human (Maines and Trakshel, 1993) BVR improve the quality of the assay by allowing the use

of much less enzyme and the removal of other cytosolic constituents that affect heme degradation (O'Carra and Colleran, 1969;Nakajima *et al.*, 1963).

CPR not only supplies electrons to HO-1, but also to cytochromes P450 and other ER-resident enzymes such as fatty acid desaturase (Strittmatter *et al.*, 1974), squalene monooxygenase (Laden *et al.*, 2000;Li and Porter, 2007), and cytochrome b_5 (Schenkman and Jansson, 2003). Due to the many potential interactions of CPR with other enzymes, it is important to better understand the behavior of HO-1 with CPR in a membrane environment. This requires the examination of full length HO-1, because of the inability of the soluble form to associate with the membrane (Huber, III *et al.*, 2009). The interplay between CPR and its many electron acceptors can have a significant effect on heme degradation, drug metabolism, and cholesterol synthesis as well as fatty acid metabolism.

Recently, our lab demonstrated that the C-terminal region present on HO-1 influences both membrane incorporation and the formation of a high-affinity complex between full-length HO-1 and CPR (Huber, III *et al.*, 2009). Not only does this form of HO-1 bind tightly to the membrane and CPR, but the characteristics of heme degradation by the full-length protein differ significantly from those found with the soluble enzyme, requiring re-examination of the conditions for the standard HO-1 assay. Here we report on an assay designed to examine HO-1 further in a membrane environment. The replacement of crude rat liver cytosol with purified hBVR, together with the optimization of other assay components, such as the addition of catalase, resulted in a method that accurately measures HO-1 activity for an extended period of time. Because the assay involves known concentrations of all three enzymes, each can

be effectively controlled and varied. With the development of this assay, the interactions of membrane-bound HO-1 and CPR can be more closely examined.

EXPERIMENTAL PROCEDURES

Materials — Heme, NADPH, EDTA, glycerol, dilauroylphosphatidylcholine (DLPC), catalase from bovine liver, superoxide dismutase (SOD), hydrogen peroxide (30%) (H_2O_2), trichloracetic acid, ferrous ammonium sulfate, potassium thiocyanate, and bovine serum albumin (BSA) were purchased from Sigma. The biliverdin used in biliverdin reductase quantification was purchased from Frontier Scientific. All spectrophotometric analyses of HO-1 activity were performed on a SpectraMax M5 plate reader from Molecular Devices (Sunnyvale, CA).

HO-1 expression and purification – Recombinant, full-length, human HO-1 was purified and quantified as previously described (Huber, III and Backes, 2008;Huber, III and Backes, 2007). The HO-1 preparation contains an R254K mutation to enhance protein stability during the GST-tag removal procedure involving the protease thrombin. Aliquots of the apoprotein were stored at -80°C.

CPR expression and purification—Full-length human CPR was expressed and purified from *E. coli* according to a previously described method (Marohnic *et al.*, 2006), with minor modifications. Following the ultracentrifugation for 1 h at 100,000 x *g*, the detergent-solubilized protein fraction (supernatant) was applied to 2',5'-ADP-Sepharose 4B (GE Healthcare), and washed extensively with detergent-free buffer until the $A_{280nm}^{in} = A_{280nm}^{out}$. A further wash step containing 5 mM adenosine was used prior to elution of

CPR using dual 2'AMP gradients (0-500 µM & 0.5-5 mM), which effectively separated full-length from N-terminal truncated products. The protein migrated as a single band by SDS-PAGE after Coomassie blue staining. Pooled fractions were concentrated using Centriprep (Millipore) centrifugal concentrators with 30 kDa molecular weight cutoff. Specific content was measured by absorbance of oxidized protein-bound flavins in the visible region of the spectrum using an extinction coefficient of 21.4 $mM^{-1}cm^{-1}$ and by the Micro BCA method according to the manufacturer's protocol (Pierce, Rockford, IL). Each assay was performed in triplicate using multiple dilutions. Aliquots were stored under liquid N_2.

Isolation of BVR from rat liver cytosol and expression and purification of human BVR – Rat liver cytosol provided the source of partially purified rat BVR and was prepared according to previously described methods (Tenhunen *et al.*, 1970;Kutty and Maines, 1981). Recombinant human BVR was prepared as follows. The human BVR (hBVR) cDNA was amplified by PCR using primers 5'-GAGACCCATATGAATACAGAGCCCGAG-3' and 5'-GACACTCGAGTGGAAGTGCTACATCACCT-3' and cloned between the *Nde*I and *Xho*I sites of vector pET-19b (Novagen, Inc) resulting in the addition of an N-terminal His_{10} tag. For His-hBVR purification, pET19b/hBVR was transformed into BL21 (DE3) cells and an individual transformant was cultured for 20 h at 37°C in 400 ml of 2xYT media. Cells were pelleted by centrifugation at 7,000 x g for 10 min at 4°C and the pellet was resuspended in 10 ml of Binding Buffer (20 mM Tris-HCl, pH 8.0, containing 5 mM imidazole and 0.5 M NaCl). The cell suspension was supplemented with 1.5 mg of

lysozyme, 0.5 mM benzamadine and 0.2 mM PMSF and incubated on ice for 15 min after which Triton X-100 was added to a final concentration of 0.1% (v/v). The cells were sonicated (3x 6 sec) and then centrifuged 15,000 x g for 15 min. The supernatant was collected and subjected to Ni^{2+} affinity chromatography as previously described (He *et al.*, 2001). Briefly, the cell extract was loaded on a Ni^{2+}-agarose column (2-ml bed volume) equilibrated in Binding Buffer. After loading, the column was washed with 10 bed volumes of Binding Buffer, followed by 10 volumes of Wash Buffer (20 mM Tris-HCl, pH 8.0, containing 60 mM imidazole and 0.5 M NaCl). His-hBVR was eluted with Elution Buffer (20 mM Tris-HCl, pH 8.0, containing 250 mM imidazole and 0.5 M NaCl) and collected in 0.5 ml fractions. Appropriate fractions were pooled and dialyzed for 2-4 h at 4^0C against 2 changes of 250 ml of His-tag Dialysis Buffer (20mM Tris-HCl, pH 8.0, containing 40 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, and 20% glycerol). Protein concentration was determined by the Bradford assay and purity was determined by SDS-PAGE.

BVR activity assay – The specific activity of partially purified rat BVR and hBVR was analyzed by monitoring the conversion of biliverdin to bilirubin. Assay conditions were carried out as previously described (Kutty and Maines, 1981), with minor modifications. The assay buffer was 100 mM KPO_4, pH 7.4, supplemented with 0.25 mg/ml fatty acid-free BSA. The reaction was performed in a Spectramax M5 plate-reader at 37 °C for approximately 5 minutes. The addition of NADPH (0.5 mM) initiated the reaction and buffer was added to the reference wells. Enzyme activity was calculated by the increase in absorbance at 450 nm using the extinction coefficient 53 mM^{-1} cm^{-1}. One

unit of BVR was defined as the amount of enzyme required to metabolize 1 nmol hr^{-1} biliverdin.

Reconstitution of HO-1 and CPR in DLPC liposomes – Reconstituted systems (RCS) were made by preincubating HO-1 and CPR with DLPC for 2 hours at room temperature, according to our recent report describing the incorporation of HO-1 and CPR into preformed DLPC liposomes (Huber, III *et al.*, 2009). A stock suspension of DLPC liposomes was prepared to 8 mM in 50 mM potassium phosphate buffer, pH 7.25, supplemented with 20% glycerol, 0.1 M NaCl, and 5 mM EDTA (DLPC sonication buffer), and sonicated in a waterbath until clear (approximately 15-20 minutes). In the initial BVR comparison experiments, as well as the BSA and BVR titration experiments, the DLPC: HO-1 ratio was 160:1, which was found to be optimal for certain P450 enzymes (Causey *et al.*, 1990). For the DLPC titration, RCS prepared with various DLPC:HO-1, ranging from 0:1 to 1280:1, were assayed. RCS for the final CPR titration were prepared in 350:1 DLPC: HO-1, which was determined to be optimal. The final concentration of HO-1 in all RCS was 0.05 µM, unless otherwise stated. CPR levels for all experiments were held constant at 0.01 µM (1:5 CPR:HO-1) and 0.2 µM (4:1 CPR:HO-1), with the exception being the CPR titration where the concentrations ranged from 0- 0.1 µM.

Optimization experiments – HO-1 activity was examined using the compound bilirubin assay as previously reported (Maines, 1996;Huber, III and Backes, 2007) with minor modifications. Bilirubin formation was monitored at 464-530 nm and the enzyme activity

was calculated using the extinction coefficient 40 mM^{-1} cm^{-1}. In general, RCS were prepared and allowed to preincubate for 2 hours at room temperature prior to the addition of other assay components as described above. The final volume for all assays was 0.1 ml. All activity assays were allowed to preincubate at 37°C for 2 minutes prior to the addition of 0.5 mM NADPH to initiate the reaction. Each reaction was performed in triplicate and the activity was expressed as nmoles of bilirubin per hour per nmol HO-1 (nmol hr^{-1} $nmol^{-1}$).

In the initial BVR comparison study, the RCS were mixed into 100 mM KPO_4, pH 7.4, 0.25 mg/ml fatty-acid free BSA, 15 µM heme, and ~ 100 U/ml of both BVR sources, assayed separately. For the pure BVR studies, the RCS were mixed with the same assay components, but the levels of BVR were varied from 0 – 0.1 µM. The BSA titration was performed by adding increasing levels of fatty acid-free BSA (0 – 2.0 mg/ml) into the 100 mM KPO_4 assay buffer prior to the addition of the RCS. When superoxide dismutase (SOD) and catalase were titrated separately into the reactions, the concentrations were 0 – 1.25 U/µl and 0 – 2.5 U/µl, respectively. As mentioned above, the DLPC titration involved increasing lipid levels in the RCS; but all other assay conditions remained the same. The CPR titration was performed under the following optimized assay conditions: 350:1 DLPC:HO-1, 15 µM heme, 100 mM KPO_4, supplemented with 0.25 mg/ml fatty acid-free BSA, 0.05 µM pure hBVR, and 0.5 U/µl catalase.

Additional experiments – Exogenous hydrogen peroxide (H_2O_2) ranging from 0 – 60 µM was added to the HO-1 reactions prior to the addition of NADPH. The HO-1 and CPR

concentrations were 0.05 µM and 0.1 µM, respectively. As described above, bilirubin formation was monitored real time. In the experiments designed to measure H_2O_2 production, the HO-1 and sHO-1 concentrations used were 0.5 µM in a reaction volume of 0.2 mL. The DLPC:HO-1 was 320:1. Three CPR:HO-1 ratios were examined; including 1:5, 2:1, and 15:1. The buffer was 100 mM potassium phosphate (pH 7.4) containing 0.165 mg/mL BSA. The 30-second reactions were initiated with 0.2 mM NADPH, and were quenched by pipetting 0.15 mL of the incubation into 0.25 mL of ice-cold 3% trichloroacetic acid. H_2O_2 was detected by the colorimetric reaction with ferrous ammonium sulfate and potassium thiocyanate as previously described (Hildebrandt *et al.*, 1978).

RESULTS

Comparison of partially purified BVR from rat liver cytosol with purified, recombinant human BVR – The membrane-binding region of full-length HO-1 has a significant influence on the kinetic characteristics of the enzyme (Huber, III and Backes, 2007). These differences call into question whether conditions that are adequate for measuring the activity of soluble HO-1 can be used to examine the full length form of the enzyme. Thus, it was important to identify the components necessary and the optimal concentrations required for a reliable assay of HO-1 activity under chemically-defined conditions. As a first step, BVR acquired from two different sources was compared: the commonly used partially purified BVR from rat liver cytosol and a highly purified, recombinant human BVR prepared in our laboratory. **Figure 2** illustrates bilirubin formation at 464-530 nm in a reaction that included HO-1 (0.05 μM), saturating CPR (0.2 μM), and saturating BVR (100 U/ml). Initially, the reaction rate of bilirubin formation for both BVR samples was equivalent at approximately 650 nmol hr^{-1} $nmol^{-1}$ HO-1; however, the duration of linearity for each reaction was drastically different. The reaction in the presence of rat liver cytosol was linear for ~ 2 minutes, whereas that in the presence of purified BVR was linear for only 20-30 s. Because the reaction catalyzed by pure BVR was linear for such a brief period, measurement of the rate of bilirubin formation was more difficult, and suggested either some limitation in a substrate (*e.g.*, NADPH or heme), or degradation of a product, intermediate, or enzyme. One explanation for these results is that the rat liver cytosol contained one or more constituents that supported the formation of bilirubin for a longer period of time.

BSA titration of the HO-1 activity assay – Next, we wanted to test various conditions in an attempt to increase linearity of the pure BVR reaction. Bovine serum albumin (BSA) has been reported to stabilize BVR, protect it from dilution-dependent inactivation, and increase overall activity (Kutty and Maines, 1981). Titration studies were designed to determine the BSA concentration required for optimal bilirubin production, and also to determine if BSA affected the linearity of the time course. As shown in **Figure 3A**, BSA was required for bilirubin production at 0.05 µM HO-1 with sub-saturating (1:5 CPR: HO-1) and saturating (2:1 CPR: HO-1) levels of CPR. Interestingly, at both CPR:HO-1 ratios, 0.25 mg/ml was determined to be the optimal concentration of BSA. Above this level, BSA inhibited the reaction. The real time kinetic curves (**Figure 3B**) indicated that BSA clearly had no influence on the linearity of the reaction for both CPR concentrations, and in agreement with previous reports and **Figure 3A**, there was little to no bilirubin formation if BSA was excluded from the reaction. Conversely, when rat liver cytosol was used in the reaction, exogenous BSA was not required for activity, suggesting that the cytosol contained a certain level of an endogenous protein that could substitute for albumin (not shown); however, even in this case, optimal activity was achieved when BSA was included. Although these results indicated that BSA was required for bilirubin production and the optimal concentration was established, as shown by the real-time kinetic curves, BSA was unable to increase the length of linearity. Thus, the absence of BSA, or other putative stabilizing protein, in the pure BVR was not the cause for the very short duration of linearity of the time course.

BVR titration of the HO-1 assay – Initially, it was hypothesized that the substrate heme may be limiting in the HO-1 assay which contained pure BVR, but this was later shown not to be the case. The optimal heme concentration was 15 μM (data not shown). The apparent K_m was approximately 2 μM, which is consistent with previously reported values (Yoshinaga *et al.*, 1982a). Thus, the next set of studies focused on possible product inhibition, by biliverdin. Biliverdin, at excess concentrations, was reported to be a potent inhibitor of both HO-1 and BVR (Kutty and Maines, 1984). To examine the effect of increasing BVR levels in the HO-1 assay, 0 – 0.1 μM of pure BVR was titrated at 1:5 CPR:HO-1 and 2:1 CPR:HO-1 (**Figure 4A**). The optimal BVR level was in excess of 0.01 μM at subsaturating reductase (*i.e.*, 1:5 CPR:HO-1) and 0.025 μM for the reaction at 2:1 CPR:HO-1 - in these experiments the HO-1 concentration was 0.05 μM. If excess biliverdin was responsible for the depressed length of linearity, then the higher BVR levels would have cleared the product more rapidly, allowing for a longer linear reaction. The real time kinetic curves of the four highest BVR concentrations (**Figure 4B**) demonstrated that increased BVR concentration had no effect on the length of linearity. A spectral scan of the completed reaction showed no buildup of biliverdin (based on the Abs at 680 nm), suggesting that BVR effectively prevented accumulation of biliverdin to an inhibitory level (data not shown). These results identified the optimal BVR concentration for the HO-1 assay, but demonstrated that the curvature in the time course was <u>not</u> attributable to the accumulation of biliverdin.

Addition of superoxide dismutase and catalase to the HO-1 assay – Having demonstrated that excess BSA and BVR had no effect on the reaction time, formation of

reactive oxygen species (ROS) was probed as the cause of the depressed linearity of the time course. In the presence of certain substrates, flavoproteins, such as CPR, have a tendency to interact with molecular oxygen and produce ROS, such as superoxide (Prough and Masters, 1973;Massey, 1994). Also, hydrogen peroxide formed in the HO-1 reaction causes non-specific heme degradation and inhibition of HO-1 (Noguchi *et al.*, 1983). In order to address these possibilities, HO-1 activity was measured in the presence of scavengers of superoxide and hydrogen peroxide by titration with superoxide dismutase (SOD) and catalase, respectively. As shown in **Fig. 5A** and **5B**, the addition of increasing concentrations of SOD (0 – 1.25 U/ml) caused no change in the reaction length, regardless of the CPR concentration. However, when catalase (0-2.5 U/ml) was included, the linearity of the time course for bilirubin formation increased at both 1:5 and 2:1 CPR:HO-1 (**Figures 5C** and **5D**). Interestingly, as catalase was added, the kinetic curve began to resemble that seen when the rat liver cytosol was used as the source of BVR; suggesting that the crude cytosol may contain catalase or another agent capable of neutralizing hydrogen peroxide. These results were surprising because in many enzyme systems that require CPR, O_2^- is produced, which is detrimental at very small concentrations. Therefore, either there was negligible O_2^- production, or the O_2^- formed had no effect on the reaction. These results were consistent with those previously reported by Noguchi, *et al.* for HO-1 purified from pig spleen microsomes (Noguchi *et al.*, 1983).

Effect of H_2O_2 on the HO-1 assay – Because the addition of catalase improved the reaction linearity, we next wanted to examine the effect of exogenous H_2O_2 on HO-1

activity. As seen in **Figure 6**, the rate of bilirubin formation is severely reduced in the presence of exogenous H_2O_2. When catalase was included in the reaction as a control, the reaction was linear for a longer period of time, consistent with the data in **Figure 5**. Next, to determine the source of the H_2O_2, we measured H_2O_2 formation in the presence and absence of HO-1 at multiple CPR concentrations. As shown in **Figure 7**, HO-1 produced H_2O_2 at 1:5 and 2:1 CPR:HO-1; however, H_2O_2 levels were undetectable in the reactions containing 2:1 CPR:sHO-1 as well as those devoid of HO-1. When CPR levels were increased to 15:1, the sHO-1 reaction produced an H_2O_2 level that was comparable to the rates observed with the 2:1 CPR:HO-1 system; however, about 25% of the peroxide production resulted from that quantity of CPR alone (**Figure 7**).

Characterization of DLPC concentration in reconstituted systems – The presence of the membrane-spanning region on HO-1 is required for efficient membrane incorporation, which is achieved following a two-hour pre-incubation period at room temperature. All of the previous characterization experiments have been performed using a DLPC:HO-1 ratio of 160:1, which was found to be optimal for other membrane-bound proteins, such as cytochrome P450 (Causey *et al.*, 1990). With other assay components optimized, the goal of this study was to determine the DLPC:HO-1 ratio that resulted in the optimal HO-1 activity. When sub-saturating CPR (0.01 μM) was preincubated with 0.05 μM HO-1 in various DLPC concentrations, the optimal DLPC:HO-1 ratio was approximately 250:1 to 350:1 (**Figure 8**). Similar results were achieved at saturating CPR, but the optimal ratio was much broader and slightly increased at approximately 300:1 to 600:1.

One explanation for these results is that the increased DLPC concentration forces a higher level of dispersion among the hydrophobic proteins, allowing for more efficient HO-1/CPR complex formation. At the lower CPR concentration, the DLPC:protein ratio of 160:1 was sufficient to produce approximately 90% of the optimal HO-1 activity. When CPR was saturating (2:1), the activity at 160:1 was approximately 75-80% of that seen with higher lipid. Thus, these data indicate that reconstituted systems containing HO-1, CPR and a higher DLPC:HO-1 ratio than typically used, provides an improved environment for HO-1 activity.

CPR titration of HO-1 – The original goal of this study was to develop an assay to measure the activity of HO-1 in the membrane environment by following bilirubin formation under defined conditions that can be used at a variety of controlled CPR to HO-1 ratios. However, the rapid deviation from linearity with membrane bound HO-1 required investigation as to its cause, and a methodological modification that produced a linear response. With the addition of catalase, the reactions remained linear for at least 3 minutes at both sub-saturating and saturating CPR levels. The next experiment was to use these defined assay conditions to examine HO-1 activity as a function of CPR concentration. Reconstituted systems containing 0.05 μM human HO-1, varying levels of human CPR (0-0.1 μM) at a 320:1 DLPC:HO-1 were preincubated and HO-1 activity determined by following bilirubin formation. Other assay components included 15 μM heme, 0.05 μM pure human BVR, 0.25 mg/ml BSA, and 0.5 U/μl catalase. As shown in **Figure 9A**, all of the reactions remained linear for at least 3-5 minutes. The result of this experiment demonstrated that with all assay components optimized, HO-1

activity was monitored effectively and accurately at a variety of CPR concentrations using a defined bilirubin assay.

Figure 9B shows HO-1 activity as a function of CPR concentration. Since the reactions remained linear for at least 3 minutes, we were able to measure the initial rate of bilirubin formation over this range of CPR concentrations. As CPR concentration was increased, the activity increased and eventually saturated. The curve fit a simple one-site hyperbolic function, and the apparent K_m was calculated to be approximately 0.01 µM. Collectively, the results of the CPR titration confirmed that the newly developed HO-1 assay was functional at a variety of CPR concentrations.

DISCUSSION

While biliverdin is a direct product of the initial oxidative degradation of heme, its poor spectral characteristics limit its utility as an effective indicator of HO-1 activity. As a result, bilirubin, which is not directly produced by HO-1, is often used to characterize HO-1 activity. In the coupled assay where bilirubin formation is monitored, HO-1 activity can be more readily quantified. Saturating levels of BVR ensure that the reduction of biliverdin to bilirubin is not the limiting factor in the rate of heme degradation (Kutty and Maines, 1981). Typically, in an HO-1 activity assay, the source of BVR was not considered to be crucial, provided it was present at a saturating level. In this report, using highly purified enzyme preparations, a defined method has been developed to assay membrane-bound HO-1 activity by monitoring the rate of bilirubin formation spectrophotometrically. This new method allows for more effective characterization studies because: 1) relative levels of the constituent proteins can be varied, 2) full-length HO-1 and CPR are used in a lipid milieu, and 3) activities can be reliably determined.

When the HO-1 assay was performed with BVR from different sources, it was clear that the BVR from rat liver cytosol (partially pure BVR) contained some component to increase linearity that was not present in the purified sample. Both heme depletion and inhibition by biliverdin and/or bilirubin were eliminated as causes of the rapid degradation of the rate of reaction. BSA was also eliminated as affecting the loss of linearity with time, although it appeared to be a necessary assay component. BSA was previously reported to stabilize and increase BVR activity by as much as 70% (Kutty and Maines, 1981), possibly by removing bilirubin from the binding site of BVR.

Albumin, or something equivalent, is one component that is endogenously present in the partially purified BVR; however, because the addition of BSA had no effect on linearity of the pure sample, inhibition of the reaction by bilirubin seemed unlikely.

The use of SOD and catalase established that hydrogen peroxide was generated at high enough concentrations to lead to the curvature in the time course. This conclusion is in strong agreement with a previous report describing the ability of uncoupled HO-1 to produce large quantities of hydrogen peroxide, leading to inhibition of heme degradation using HO-1 isolated from pig microsomes (Noguchi *et al.*, 1983). In addition, the conversion of heme to biliverdin continued after the addition of catalase. Interestingly, the antioxidant effects of liver cytosol were documented in a paper criticizing the measurement of bilirubin formation as a method for measurement of heme oxygenase activity (Lodola *et al.*, 1979). This report criticized an assay that used microsomal tissues and partially purified enzymes as the sources for the assay components, due to concerns regarding spectral interference from crude cytosolic fractions. Our assay, using only purified components, avoided this potential limitation. The presence of endogenous catalase could, however, explain the kinetic differences between the HO-1 assays catalyzed by partially pure BVR from rat liver cytosol and purified hBVR.

The deviation from linearity being due to hydrogen peroxide formation was further supported by experiments showing that (1) direct addition of H_2O_2 to the assay caused a decrease in the rate of bilirubin formation, and (2) H_2O_2 was produced by the HO-1 reaction. Interestingly, H_2O_2 generation was much more efficient with the full-length form of HO-1 than with the soluble form. We were able to obtain comparable

levels of H_2O_2 formation with the soluble form when the CPR concentration was elevated to a 15:1 CPR:HO-1 ratio – the same level of reductase that is required to obtain comparable rates of bilirubin production. Therefore, it is likely that the lower rate of peroxide formation is due to the less efficient complex formed between CPR and sHO-1, and explains the difference in linearity between the full-length and soluble forms of HO-1.

The development of our assay allows for a more accurate study of HO-1 in a membrane with CPR. Multiple studies have shown a possible interaction between CPR and BVR, as well as mutual inhibition (Higashimoto *et al.*, 2005;Wang and Ortiz de Montellano, 2003); however, in our titration experiments, there was no direct evidence that increased concentrations of either protein had any influence on the other. It must also be noted that a majority of the previous work involving HO-1, CPR, and BVR was performed with soluble enzymes. With HO-1 and CPR reconstituted into a membrane milieu, the lipid may be sequestering the two proteins and altering the ability of BVR to compete with CPR in this reaction. Another possibility is that membrane-bound HO-1 has a different conformation than sHO-1, effectively changing the way biliverdin is released from the active site. Because HO-1 and CPR are membrane-bound proteins, the presence of membrane hydrophobic milieu (DLPC or ER membrane) may alter the mechanism by which cytosolic BVR metabolizes its substrate biliverdin to bilirubin. With the development of this assay, these questions can be addressed in a lipid environment using full-length proteins. Future studies will be designed to answer questions regarding potential interactions among the three enzymes.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. **Heme degradation pathway**. Following an obligatory interaction with Cytochrome P450 reductase (CPR), heme oxygenase-1 (HO-1) initiates the oxidative degradation of heme to biliverdin, carbon monoxide (CO), and Fe^{2+}. Biliverdin is further metabolized to the lipophilic product, bilirubin, by biliverdin reductase (BVR). Although HO-1 and CPR exist bound to the endoplasmic reticulum membrane, BVR is present in the cytosol.

Fig. 2 **Bilirubin formation catalyzed by biliverdin reductase (BVR) from two different sources.** Recombinant**,** human BVR was purified to apparent homogeneity, and rat liver cytosol served as a partially purified source of BVR. *A,* Real time curves from 0-300 seconds of bilirubin formation catalyzed by partially pure BVR (*solid line)* and pure BVR (*dashed line).*

Fig. 3 **BSA is required for optimal bilirubin production at sub-saturating and saturating CPR levels.** *A,* BSA titration of a bilirubin assay at 1:5 CPR:HO-1 ((□, *dashed line*) and 2:1 CPR:HO-1 ▲, *solid line*). *B*, Real time kinetic traces of bilirubin production with 0 mg/ml BSA: 1:5 CPR:HO-1 (—), 0 mg.ml BSA, 2:1 CPR:HO-1 (— ·· —),0.25 mg/ml BSA: 1:5 CPR:HO-1 (— · —), and 0.25 mg.ml BSA, 2:1 CPR:HO-1 (- - -). HO-1 concentration was 0.05 µM for all assays. Values in Panel *A* represent the mean +/- S.E. of n=3.

Fig. 4 **BVR titration and real time curves of bilirubin production.** *A,* BVR ranging from 0 – 0.1 µM were analyzed to determine the optimal concentration for bilirubin production at sub-saturating and saturating CPR concentrations: 1:5 CPR:HO-1 (□) and 2:1 CPR:HO-1 (▲). *B,* Real time bilirubin assay curves of the four highest BVR concentrations (0.01-0.1 µM). HO-1 concentration was 0.05 µM for all assays. Values in Panel *A* represent the mean +/- S.E. of n=3.

Fig. 5 **Effect of SOD and catalase on the linearity of the bilirubin formation real time activity curves.** *A and B,* Increasing amounts of superoxide dismutase (0, .00025, 0.00125, 0.00625, 0.025, 0.25, 0.625, and 1.25 U/µl) were added to the reaction mixture containing 1:5 (*A*) and 2:1 (*B*) CPR: HO-1. *C and D*, Catalase (0, 0.00025, 0.00625, 0.025, 0.25, 0.625, 1.25, and 2.5 U/µl) was titrated into the 1:5 (*C*) and 2:1 (*D*) CPR:HO-1 reactions. HO-1 concentration was 0.05 µM for all reactions.

Fig. 6 **Effect of hydrogen peroxide on HO-1 activity.** H_2O_2 was added to a series HO-1 reactions containing 2:1 CPR: HO-1 in the absence of catalase. H_2O_2 concentrations included 0 µM (Δ), 5 µM (♦), 15 µM (○), 30 µM (■), and 60 µM (◊). Bilirubin formation in the presence of catalase and no exogenous H_2O_2 was included for comparison (dashed line). Each point represents the average of 3 determinations.

Fig. 7 **Generation of hydrogen peroxide by full-length and soluble HO-1.** H_2O_2 produced by HO-1 and sHO-1 was measured at various CPR:HO-1. The HO-1/sHO-1 concentration was 0.5 µM. Low, mid, and high CPR corresponds to a DLPC/CPR

reconstituted system (devoid of HO-1) using the same CPR concentrations as those used in the 1:5, 2:1, and 15:1 conditions."N.D." indicates that hydrogen peroxide was not detected. Each bar represents the mean ± S.E.M. for 3 determinations.

Fig. 8 **DLPC titration of HO-1 in RCS**. DLPC levels were varied from 0 – 1200 : 1 DLPC:HO-1. Following the 2hr incubation period at room temperature, the RCS were assayed by monitoring bilirubin formation at 464-530 nm. Shown here are 1:5 CPR:HO-1 (■) and 2:1 CPR:HO-1 (∆). The HO-1 concentration was 0.05 µM for all assays. Values represent the mean +/- S.E. of n=3.

Fig. 9 **Effect of CPR concentration on HO-1 activity.** *A,* Time course for bilirubin formation. CPR concentrations included: 0 µM (●), 0.0015 µM (○),0.003 µM (■), 0.00625 µM (□), 0.0125 µM (▲), 0.025 µM µM (∆), 0.05 µM (♦), and 0.1µM (◊). *B,* The rate of bilirubin formation as a function of CPR concentration. All assays contained 0.05 µM HO-1. Values in Panel *B* represent the mean +/- S.E. of n=3.

Figure 1

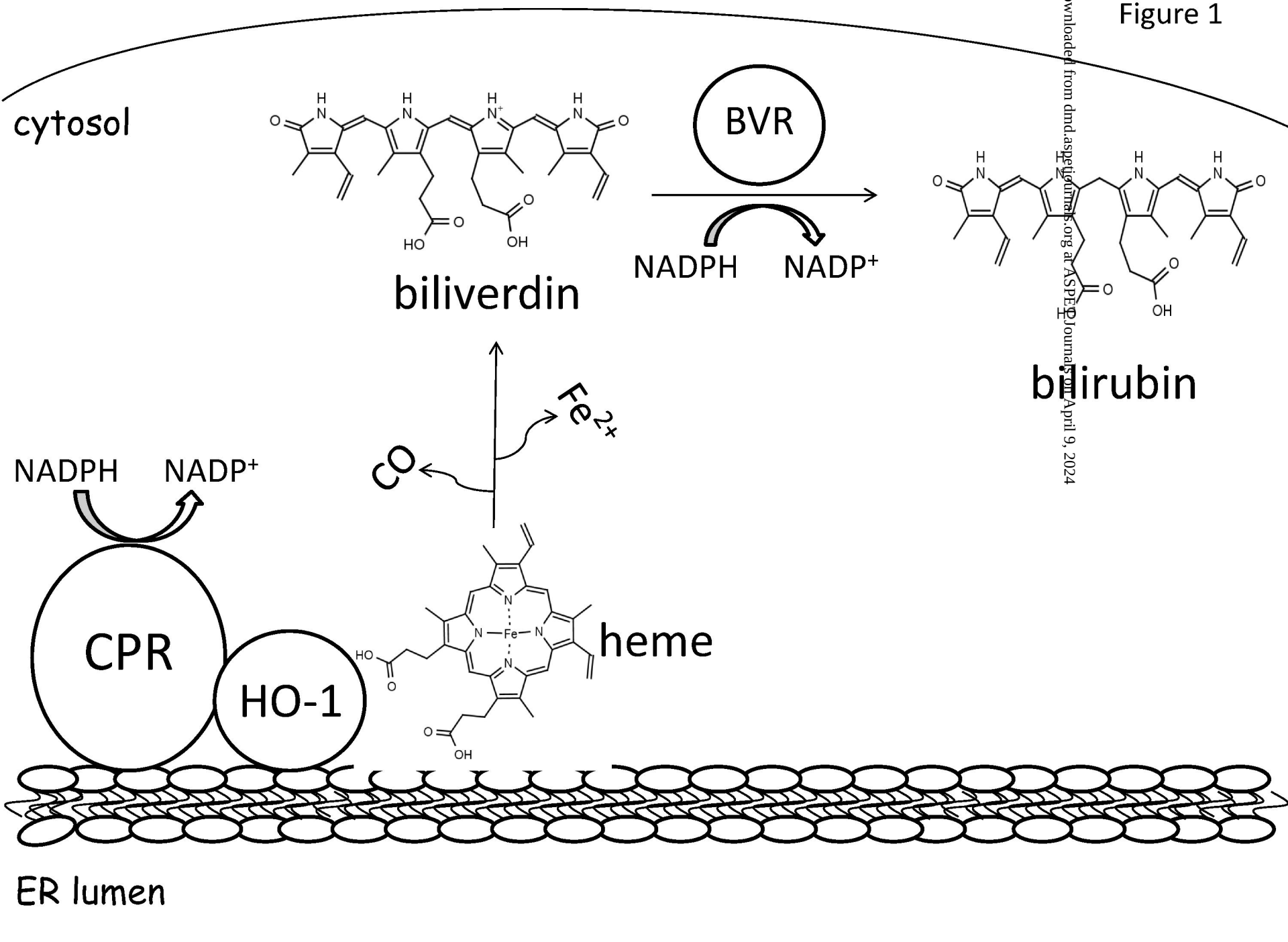


Fig 2

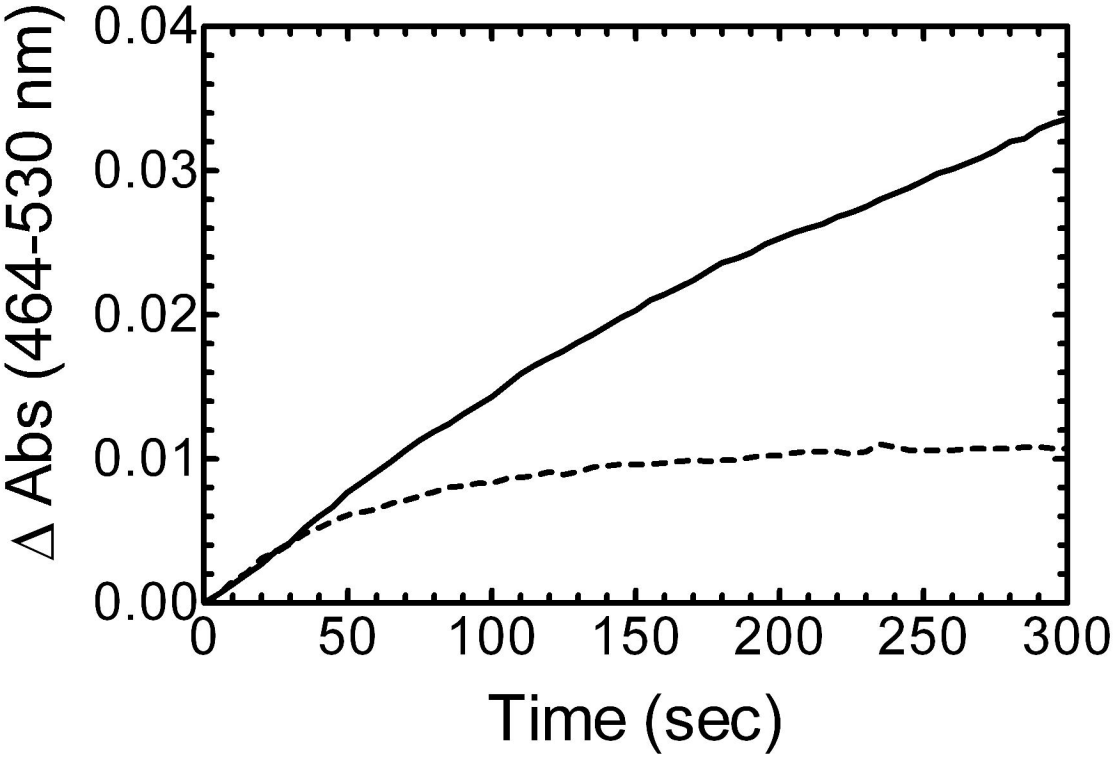

0.04
0.03
0.02
0.01
0.00
Δ Abs (464-530 nm)
0
50
100
150
200
250
300
Time (sec)

Fig 3A

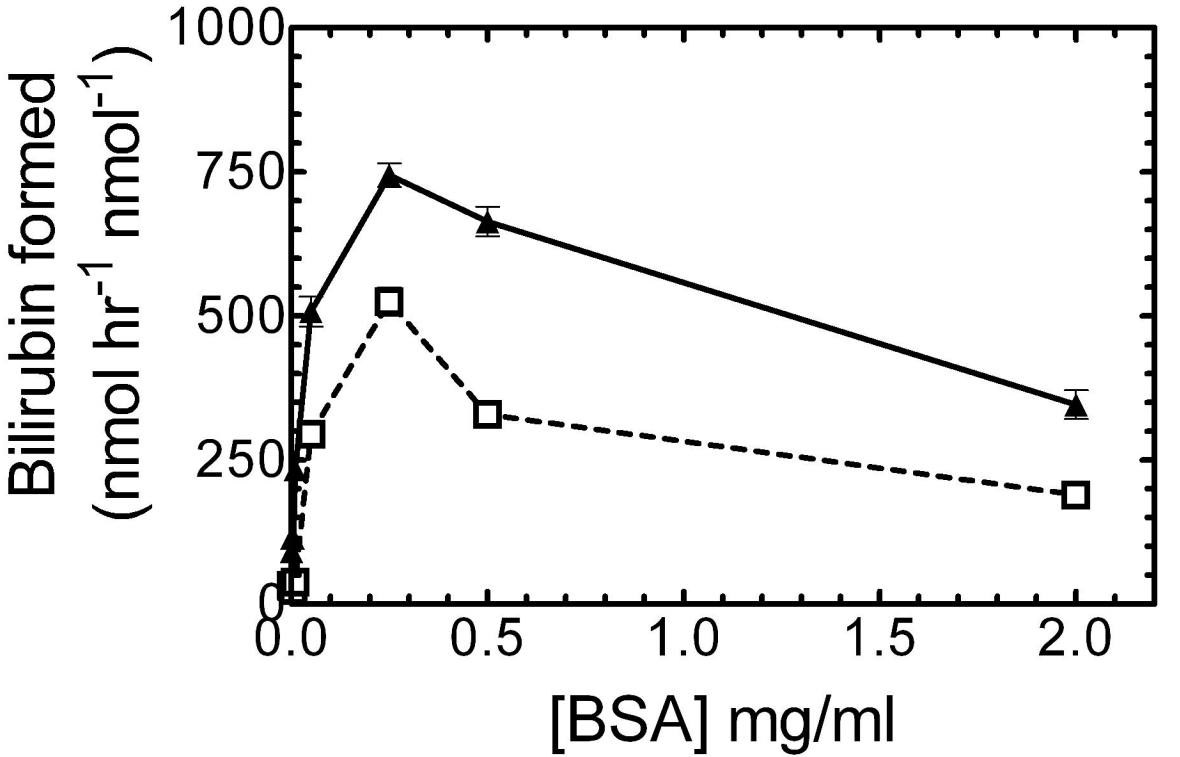

Bilirubin formed
(nmol hr-1 nmol-1)
1000
750
500
250
0
0.0
0.5
1.0
1.5
2.0
[BSA] mg/ml

Fig 3B

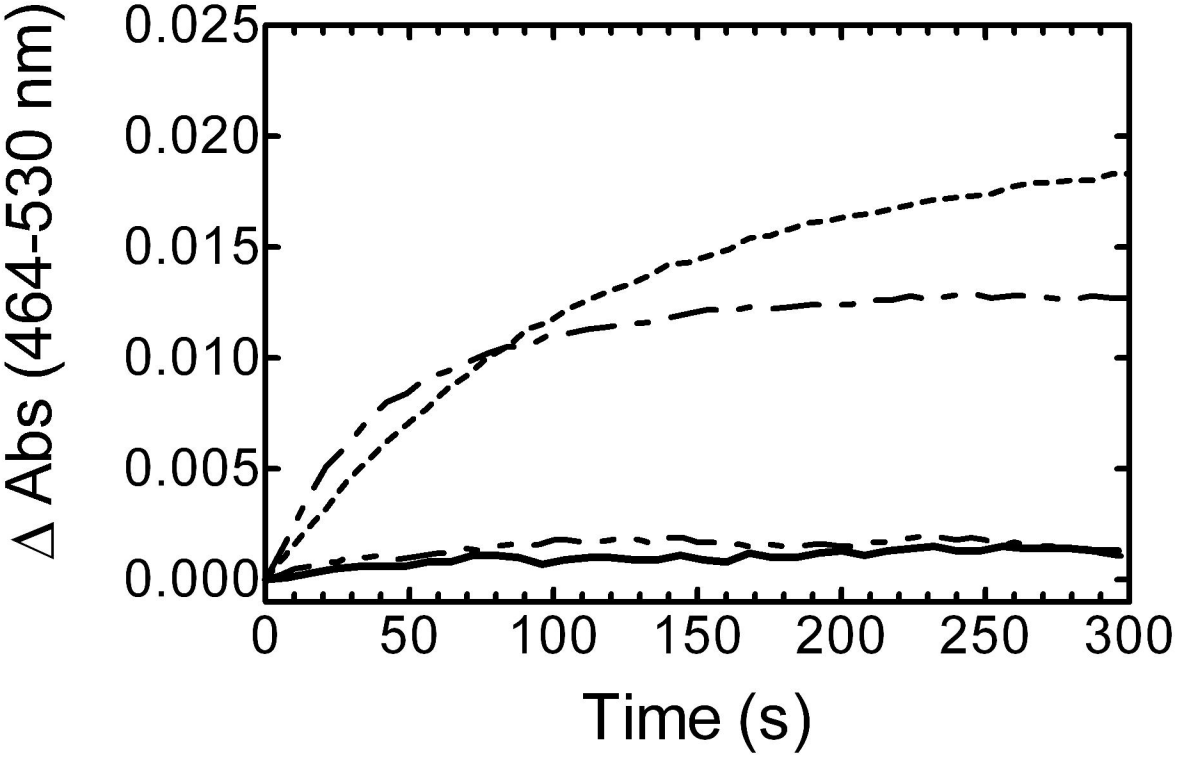

Δ Abs (464-530 nm)
0.025
0.020
0.015
0.010
0.005
0.000
0
50
100
150
200
250
300
Time (s)

Fig 4A

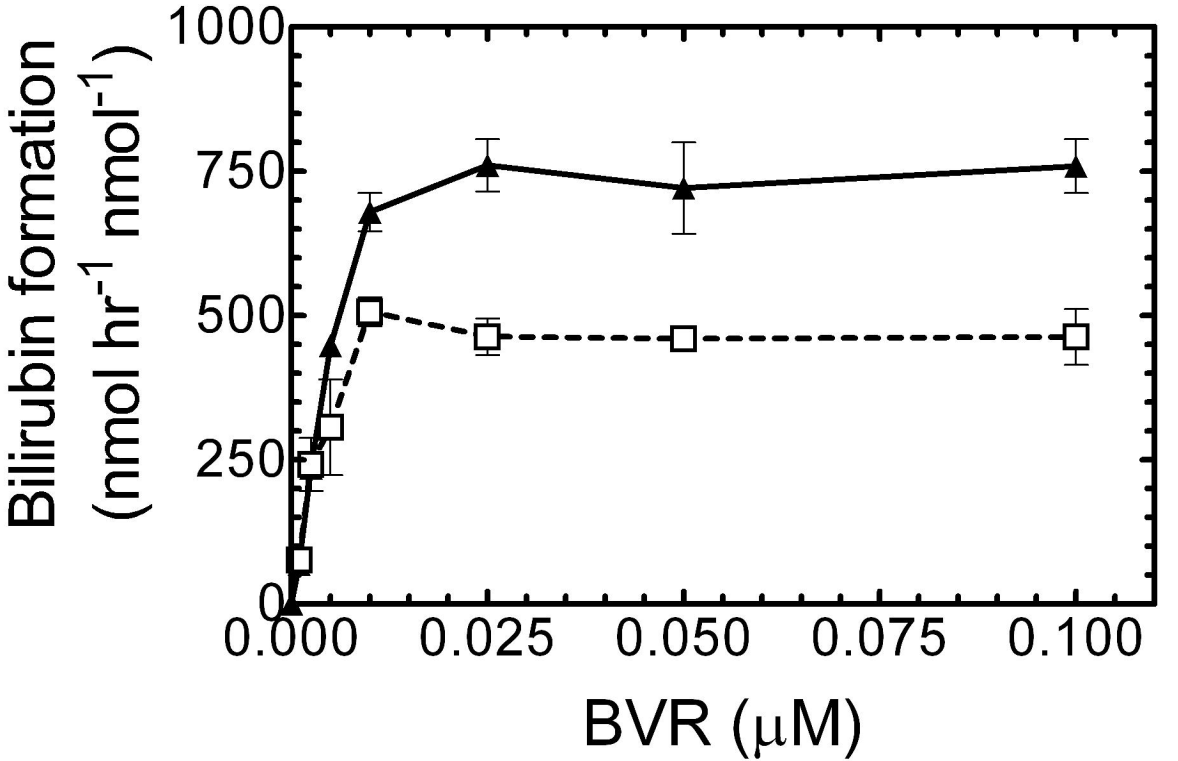

Bilirubin formation
(nmol hr-1 nmol-1)
1000
750
500
250
0
0.000
0.025
0.050
0.075
0.100
BVR (μM)

Fig 4B

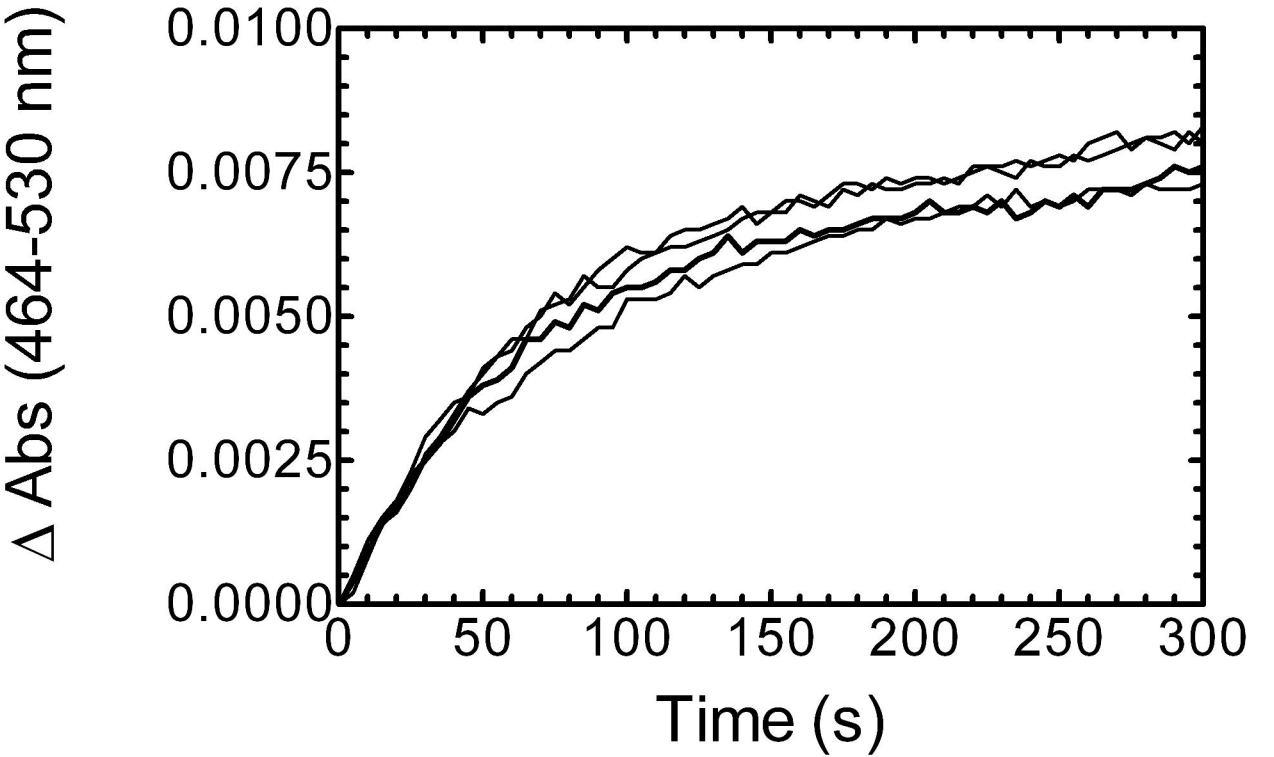

0.0100
0.0075
0.0050
0.0025
0.0000
Δ Abs (464-530 nm)
0
50
100
150
200
250
300
Time (s)

Fig 5A

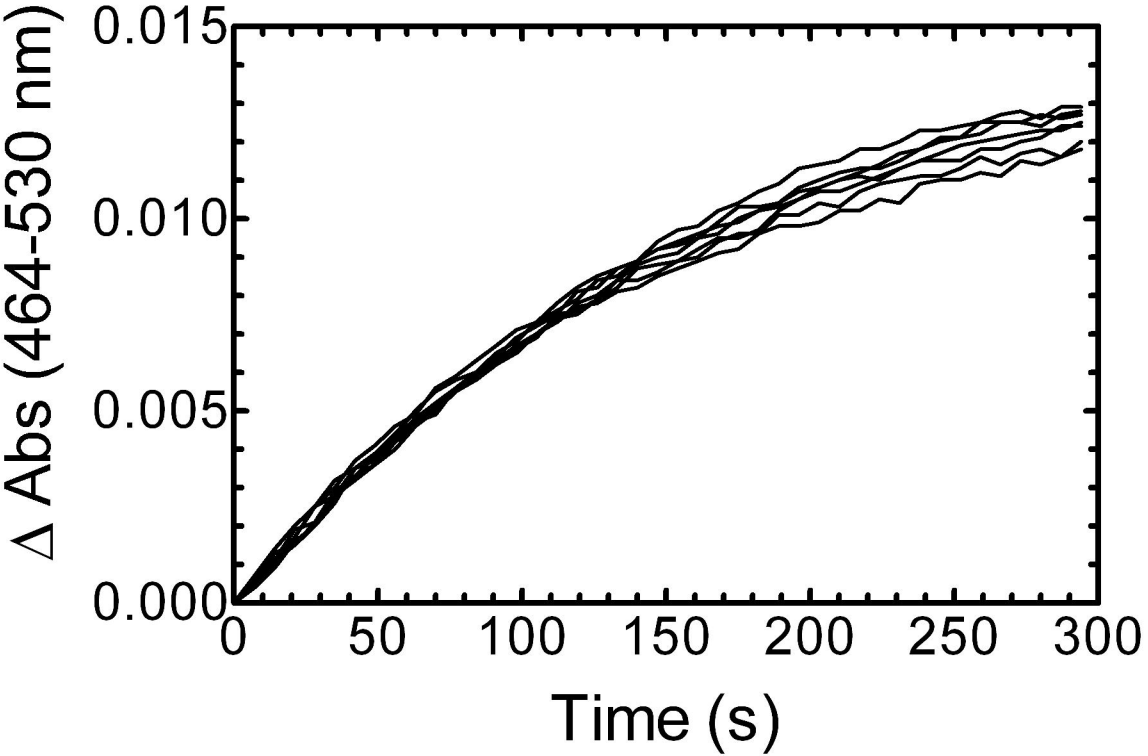

0.015
0.010
0.005
0.000
Δ Abs (464-530 nm)
0
50
100
150
200
250
300
Time (s)

Fig 5B

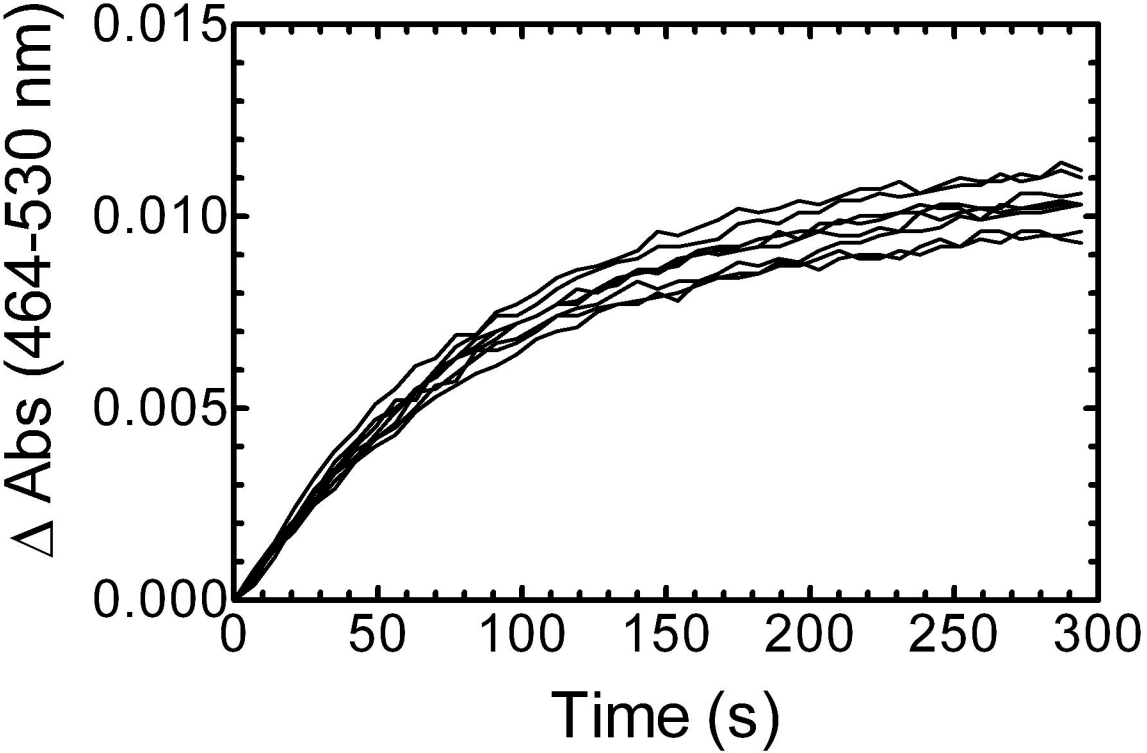

0.015
0.010
0.005
0.000
Δ Abs (464-530 nm)
0
50
100
150
200
250
300
Time (s)

Fig 5C

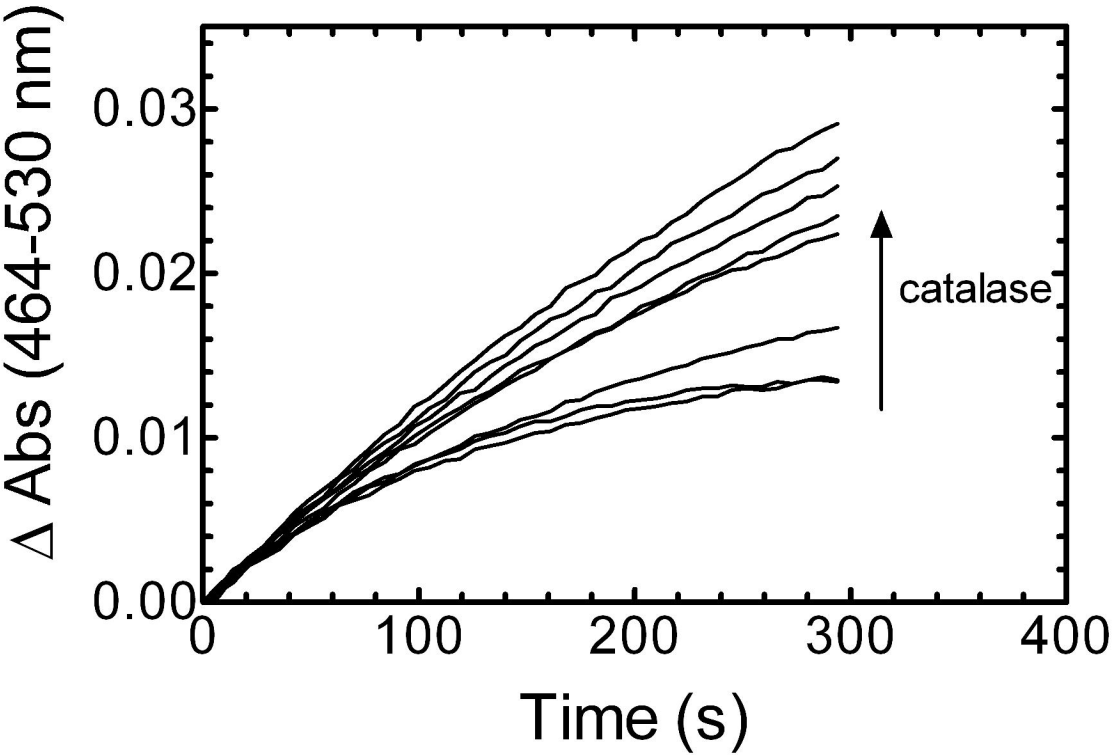

Δ Abs (464-530 nm)
0.00
0.01
0.02
0.03
0
100
200
300
400
Time (s)
catalase

Fig 5D

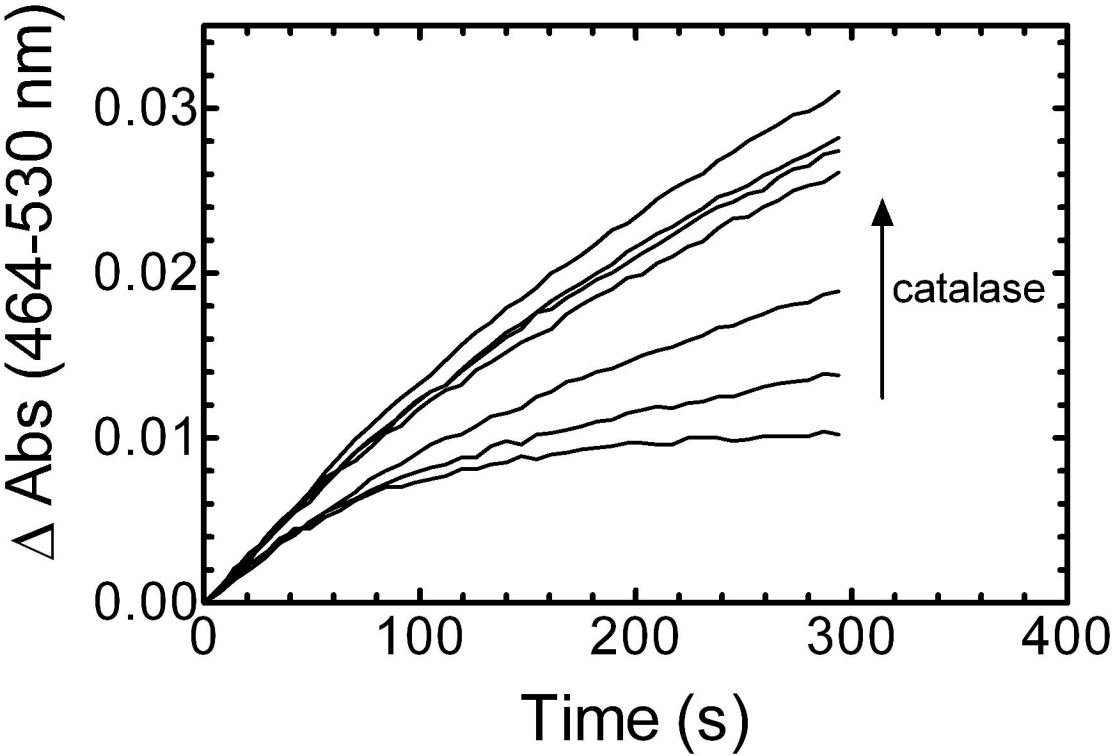

Δ Abs (464-530 nm)
0.00
0.01
0.02
0.03
0
100
200
300
400
Time (s)
catalase

Fig 6

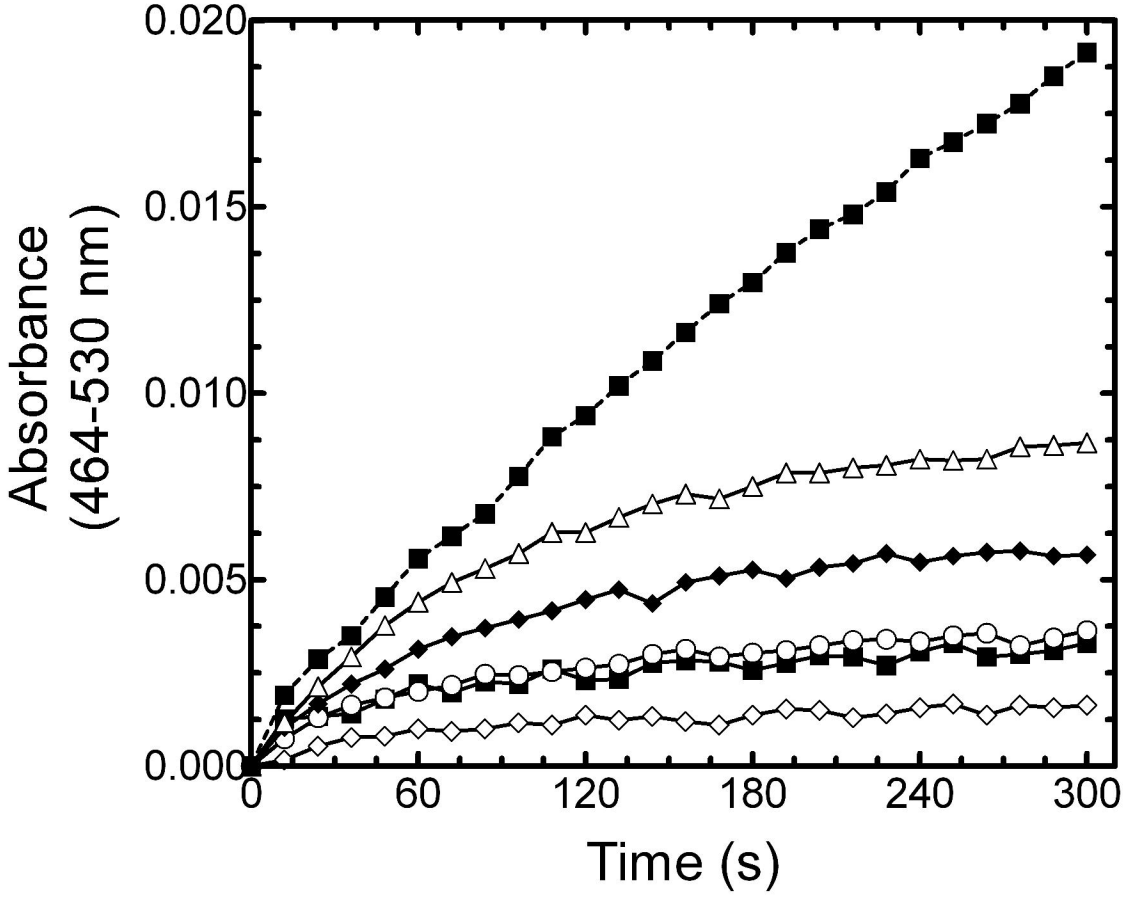

0.020
0.015
0.010
0.005
0.000
Absorbance
(464-530 nm)
0
60
120
180
240
300
Time (s)

Fig 7

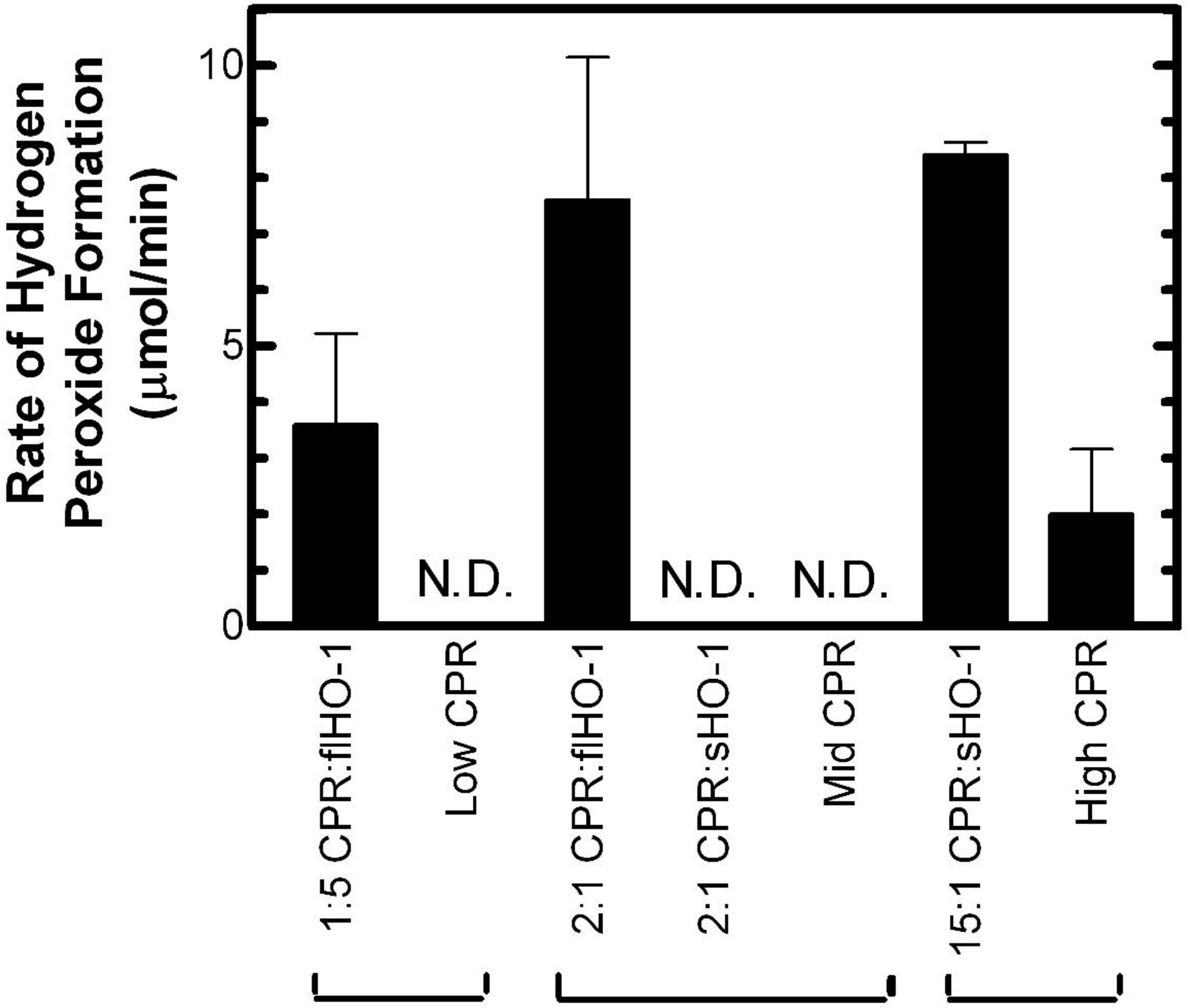

Rate of Hydrogen Peroxide Formation (μmol/min)
10
5
0
N.D.
N.D.
N.D.
1:5 CPR:flHO-1
Low CPR
2:1 CPR:flHO-1
2:1 CPR:sHO-1
Mid CPR
15:1 CPR:sHO-1
High CPR

Fig 8

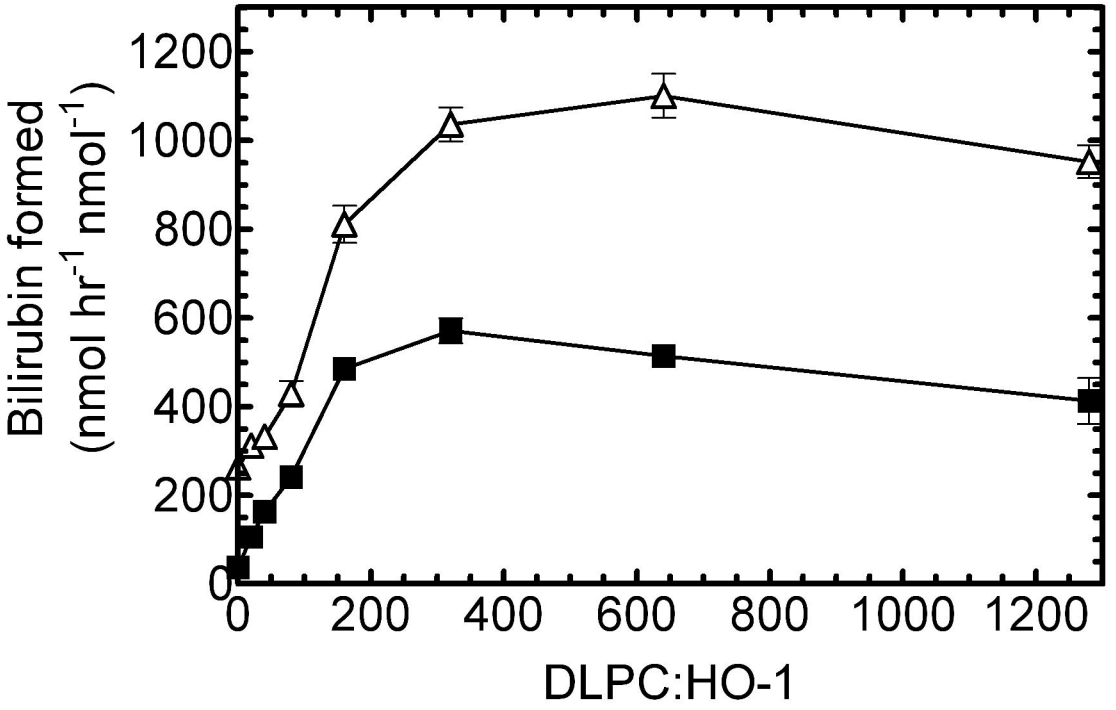

Bilirubin formed
(nmol hr-1 nmol-1)
0
200
400
600
800
1000
1200
DLPC:HO-1

Fig 9A

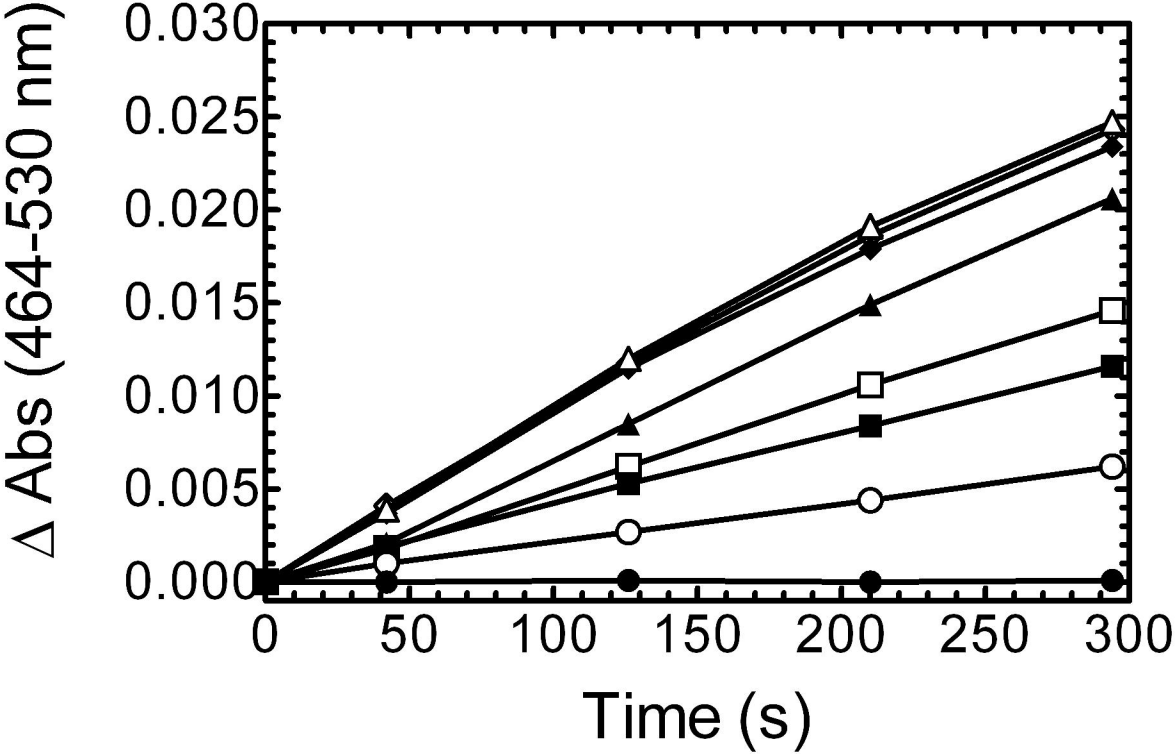
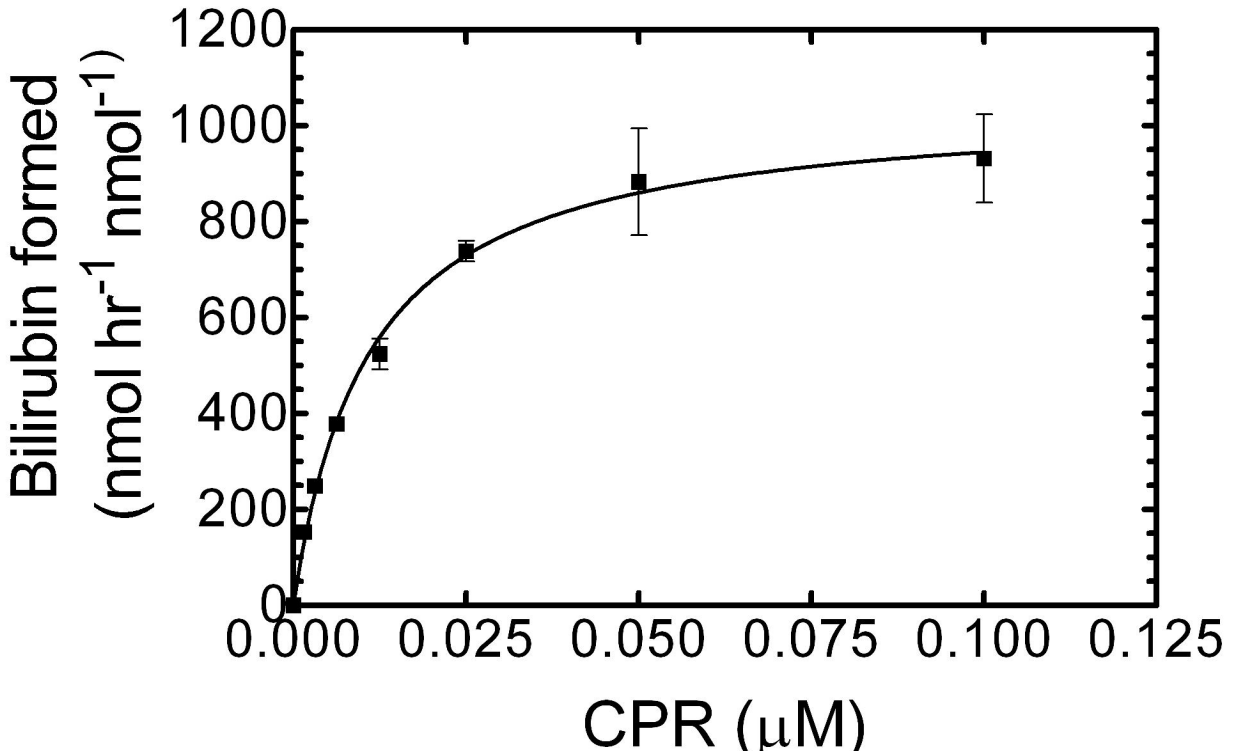

Δ Abs (464-530 nm)
0.030
0.025
0.020
0.015
0.010
0.005
0.000
0
50
100
150
200
250
300
Time (s)

Fig 9B


Bilirubin formed
(nmol hr-1 nmol-1)
1200
1000
800
600
400
200
0
0.000
0.025
0.050
0.075
0.100
0.125
CPR (μM)