Cloning and overexpression of rat kidney biliverdin IX α reductase as a fusion protein with glutathione S-transferase: stereochemistry of NADH oxidation and evidence that the presence of the glutathione S-transferase domain does not effect BVR-A activity

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Native biliverdin IX α reductase (BVR-A) is a monomer of molecular mass 34 kDa. We have developed an expression vector that allows the isolation of 40 mg of a glutathione S-transferase (GST)–BVR-A fusion protein from 1 litre of culture. The fusion protein (60 kDa) behaves as a dimer on gel filtration (120 kDa), so that we have artificially created a BVR-A dimer. The recombinant rat kidney enzyme exhibits pre-steady-state ‘burst’

kinetics that show a pH dependence similar to that already described for ox kidney BVR-A. Similar behaviour was obtained in the presence and absence of the GST domain both for the burst kinetics and during initial-rate studies in the presence and absence of albumin. The stereospecificity of the BVR-A-catalysed oxidation of [4-³H]NADH, labelled at the A and B faces, was shown to occur exclusively via the B face.

INTRODUCTION

The haem catabolon produces bilirubin IX α (BR) via the intermediate bile pigment biliverdin IX α (BV). Bilirubin requires conjugation with glucuronic acid before excretion in the bile [1]. Indeed it is the delayed expression of the conjugating enzyme bilirubin glucuronosyltransferase at birth that results in so-called physiological or neonatal jaundice [2]. Elevated levels of BR, particularly if they exceed the binding capacity of serum albumin [3], are toxic, inhibiting respiration and a large number of key enzymes. To avoid neural toxicity in neonatal jaundice, serum BR is lowered by exposure to visible light. This causes photoisomerization and enhanced excretion of the photoisomers of BR [4]. In severe cases of hyperbilirubinaemia, serum bilirubin is lowered by an exchange transfusion [5]. Recent evidence that BR functions as an antioxidant *in vitro* [6–8] suggests that a more balanced view might be needed concerning levels of BR in serum. The rate-limiting step in haem catabolism is generally regarded as the haem oxygenase (HO)-catalysed formation of BV from haem (but see [9] for evidence that under some conditions this might not hold). HO exists in two forms; one of these (HO-1) is up-regulated by a wide variety of cellular insults [10]. This suggests that the haem catabolon might function as a stress pathway [11]. CO, which is formed uniquely by the activity of HO, has been suggested to function in the central nervous system as a neural messenger [12,13]. Interest in the possible antioxidant role for BR has been further stimulated by reports that low serum levels of BR are correlated with coronary artery disease [14,15] and that BR protects low-density lipids from lipid peroxidation [16]. In addition, Willoughby and co-workers have recently suggested that HO-1 might be a target for anti-inflammatory drugs because the modulation of ‘BR production capacity’ suggests that increases in HO-1 activity down-regulate

the inflammatory response in a carageenin-induced model of acute inflammation [17]. Although there is a considerable body of evidence showing increases in HO-1 mRNA, HO-protein levels and HO-activity, there is less evidence that any particular pool of haem is mobilized for degradation, although several groups of workers have shown that induction of HO in the liver is often accompanied by decreases in cytochrome P450 (reviewed in [11]). Although in some species (for example the horse) the steady-state level of BR increases during stress [18], this is not generally so. It is likely that modest elevations of BR might not be detected in serum if BR-binding proteins, such as the glutathione S-transferases (GSTs) in liver and kidney, act as a thermodynamic sink. Further work is required in order to demonstrate clearly that there is an increase in flux through the haem catabolon during stress. In model systems *in vitro*, BR oxidation has been shown to produce stoichiometric amounts of BV; thus biliverdin IX α reductase (BVR-A) might function to maintain the level of a physiologically important antioxidant.

Although some kinetic studies have been performed on BVR-A there has been little structural work, owing to the relatively low abundance of this enzyme. This is somewhat surprising as this enzyme represents a logical pharmacological target for modulating BR levels in neonatal jaundice and during inflammation. BVR-A is subject to potent substrate inhibition [19,20] and this is probably modulated *in vivo* by GSTs [21]. There has been no detailed study of the reaction mechanism at physiological pH in the absence of BV-binding proteins. We have reported the steady-state mechanism for the ox kidney enzyme at pH 9 in the absence of BV-binding proteins [22]. This has not been possible with the rat or salmon enzymes, which have half-lives of only a few minutes at this pH [23]. Although various authors have reported K_m values for BV, the initial rates are generally measured in the presence of BSA, which profoundly influences the apparent

Abbreviations used: ADH, alcohol dehydrogenase; BR, bilirubin IX α ; BV, biliverdin IX α ; BVR-A, biliverdin IX α reductase; GDH, glutamate dehydrogenase; GST, glutathione S-transferase; HO, haem oxygenase.

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rates observed and certainly increases the apparent K_m for bilirubin. Although it is tempting to ascribe the effect of BSA to simple sequestration of substrate, detailed work in our laboratory suggests that this is not the only function ([20], and E. M. Rigney, O. Ennis, G. Elliot and T. J. Mantle, unpublished work). *In vivo* it is likely that intracellular BV-binding proteins, particularly the GSTs, have a role analogous to that of BSA *in vitro* [21].

We have reported some preliminary pre-steady-state experiments with the ox kidney enzyme that showed a pH-dependent burst [24] but were hampered by the lack of enzyme. Our preparation of the ox kidney enzyme [25] yields 1 mg/kg of tissue after six steps. To improve the yield of BVR-A and to allow us to initiate structural studies on this enzyme, we have constructed pGEX.BVR, an expression vector, which allows up to 45 mg of a fusion protein between GST and rat kidney BVR-A to be isolated from 1 litre of culture with a single affinity step. This has also allowed us to determine the stereospecificity of NADH oxidation by the enzyme. Interestingly the presence of the GST domain does not affect the activity of the enzyme in stopped-flow and initial-rate experiments. Details of these experiments are presented here.

MATERIALS AND METHODS

Materials

pGEX.KG was a gift from K. L. Guan and J. E. Dixon [26]. Primers were obtained from the Oligonucleotide Synthesis Service (Biotechnology Building, Trinity College Dublin, Dublin, Ireland). *Taq* polymerase was purchased from Promega, and a rat kidney λ gt11 cDNA library from Clontech. The glutathione–Sephacryl S-200 column was synthesized by the method of Simons and Van der Jagt [27] by coupling glutathione to epoxy-activated Sepharose.

Construction of pGEX.BVR

Oligonucleotides corresponding to the 5' and 3' ends of the rat kidney BVR-A sequence reported by Fahkrai and Maines [28] were designed so as to each include an *Nco*I restriction site (underlined): forward primer, 5'-G ACT GAA GAG ACC GCC ATG GAT GCC GAG C-3'; reverse primer, 5'-GAC ATG TTC CAT GGT AAG AGC TAA AGG TGA-3'. The bold ATG is the start codon. These primers were used to amplify BVR cDNA from a rat kidney λ gt11 cDNA library. The PCR product was digested with *Nco*I, and electrophoresed on a 1% (w/v) agarose gel. The 960 bp product was excised as a band from the gel and purified with GeneClean. The expression vector pGEX.KG was digested with *Nco*I; the restriction enzyme was then heat-inactivated. The linearized vector was treated with calf intestinal alkaline phosphatase and was also purified from a 1% (w/v) gel. Ligations were performed at 16 °C overnight with T4 DNA ligase. The ligated DNA was transformed into competent *Escherichia coli* TG1 cells. Candidate clones were analysed for BVR-A expression by sonicating overnight cultures and then incubating the suspension with 20 μ M BV/705 μ M NADH/37 μ M BSA in 100 mM sodium phosphate, pH 7.2. Enzyme expression was detected by the development of a yellow colour (BR), which was confirmed as bilirubin by treatment with diazotized sulphanilic acid [29].

Purification of GST–BVR-A

An overnight culture (10 ml; *E. coli* TG1 cells transformed with pGEX.BVR-A) was diluted into 1 litre of fresh Luria–Bertani

medium containing IPTG (0.2 mM) and incubated overnight at 37 °C. The cells were collected by centrifugation and washed in 10 mM sodium phosphate, pH 7.2. Lysozyme (200 μ g/ml), DNase (50 μ g/ml) and RNase (50 μ g/ml) were added and the suspension was sonicated for 5 min with on/off periods of 5 s each. The suspension was centrifuged at 20000 *g* for 45 min; the cleared lysate was stored on ice and the pellet subjected to a further round of sonication and centrifugation. The combined supernatants were loaded on a glutathione–Sephacryl column equilibrated with 10 mM sodium phosphate and rinsed with this buffer until the A_{280} was less than 0.05. The fusion protein was then eluted by switching the buffer to 50 mM Tris/HCl, pH 8, containing 10 mM glutathione. Gel filtration of the purified fusion protein, BSA (68 kDa), ovalbumin (45 kDa) and cytochrome *c* (12.4 kDa) was performed on a Sephacryl S-200 column (120 cm $\times$ 4.5 cm) equilibrated in 10 mM sodium phosphate, pH 7.2, containing 100 mM KCl.

Thrombin cleavage of the GST–BVR-A fusion protein

The GST–BVR-A fusion protein was incubated with various concentrations of thrombin for different periods at 4 °C and at room temperature. The cleavage products were monitored by SDS/PAGE. To remove the GST dimer the reaction mixture was loaded on glutathione–Sephacryl, from which the 'cleaved' BVR-A was eluted in the flow-through fraction. This material was separated from thrombin in some experiments by loading the wash-through material on 2',5'-ADP-Sephacryl and, after a wash with 10 mM sodium phosphate, the BVR-A was eluted with a 0–150 mM KCl gradient.

SDS/PAGE

SDS/PAGE was performed with 15% (w/v) gels [29a].

Protein sequencing

The bands of interest were blotted on a ProBlot membrane and sequenced by the Welmet Sequencing Service (University of Edinburgh, Edinburgh, U.K.).

Initial-rate kinetics

Initial-rate kinetic studies were conducted at 30 °C with the fusion protein GST–BVR-A and the 'cleaved' BVR-A. The assay mixture contained 100 mM sodium phosphate, pH 7.2, NADH (705 μ M) and various concentrations of BV. In some experiments BSA was included at a final concentration of 37 μ M. The reaction was started by the addition of enzyme (10 μ l; 1–10 μ g) and monitored at 460 nm. For monitoring enzyme activity during purification, BSA (37 μ M) was routinely included in the assay mixture. The data were fitted to equations for partial and total substrate inhibition as described previously [20].

Pre-steady-state kinetics

Pre-steady-state experiments were performed with a Hi-Tech Stopped Flow Multi Mixing Spectrometer (Model SF-61). The software used to store and analyse the data was the Hi-Tech IS-1 Rapid Kinetics software suite.

Stereochemical determination of BVR-A-catalysed NADH oxidation

[4-³H]NAD⁺ was reduced with glutamate dehydrogenase (GDH) and alcohol dehydrogenase (ADH) to produce ³H at the A and B faces respectively [30]. The GDH reaction mixture contained

20 mM sodium phosphate, pH 8.5 (1.48 ml), 100 mM glutamate (500 μ l), 50 mM NAD⁺ (8 μ l), 0.37 mM [4-³H]NAD⁺ (5 μ l; 5 μ Ci) and GDH (50 μ l; 29 mg/ml). The ADH reaction contained 20 mM sodium phosphate, pH 8.5 (1.92 ml), ethanol (60 μ l), 50 mM NAD⁺ (8 μ l), 0.37 mM [4-³H]NAD⁺ (5 μ l; 5 μ Ci) and 10 mg/ml ADH (2 μ l). The reactions proceeded at 30 °C until there was no further increase in A_{340} (10 min for GDH; 1 min for ADH). The A- and B-face-labelled [4-³H]NADH was used immediately in a BVR-A reaction. The following components were added to the reaction mixtures resulting from the use of GDH and ADH: 500 mM sodium phosphate, pH 6.5 (374 μ l), 3.85 mM BV (3.25 μ l), 1.48 mM BSA (62.5 μ l) and 60 μ l of BVR-A (32 μ g). The reaction was performed at 30 °C and the production of BR was monitored at 460 nm until completion. Controls were set up to monitor carry-over of radioactivity, where the BVR-A was replaced with 60 μ l of water. Thus the four samples, two test and two 'carry-over' controls (2.5 ml each) were transferred to screw-capped polypropylene tubes and the BR formed was extracted into 0.9 ml of chloroform to which acetic acid (160 μ l) was added. After centrifugation, 200 μ l of the chloroform layer was added to 10 ml of toluene/2,5-diphenyl-oxazole for scintillation counting.

RESULTS AND DISCUSSION

The recombinant fusion protein GST-BVR-A can be produced in large amounts by the protocol described here. The protein is homogeneous by the criteria of SDS/PAGE and runs with an

Figure 1 Initial-rate kinetics of recombinant rkBVR-A

The initial-rate kinetics of BVR-A as the GST fusion protein (a) and after thrombin cleavage (b) are shown. The assays were conducted at 30 °C as described in the text, with 14 μ g of fusion protein per assay and 1.4 μ g of 'cleaved' BVR-A, in the absence (○) or presence (●) of BSA (37 μ M). The lines are least-squares fits to the equation for total substrate inhibition.

Figure 2 Effect of pH on the pre-steady-state 'burst' kinetics of recombinant rkBVR-A

The reactions were performed as described in the text, in 100 mM sodium phosphate at the pH values indicated. The final concentrations were 50 μ M NADPH, 10 μ M BV and 3.35 μ M GST-BVR-A (□) or 3.35 μ M BVR-A (■). Data points are the means of triplicates. Also shown are data for the ox kidney enzyme (●) [24].

molecular mass of 60 kDa. On cleavage with thrombin the protein is degraded to produce BVR-A (34 kDa) and GST (24 kDa). On closer inspection, at the shorter time points (10–20 min with 0.1 unit of thrombin/100 μ g of fusion protein, or 2 h with 0.01 unit of thrombin/100 μ g of fusion protein), it is clear that the BVR-A is resolved into a doublet. N-terminal sequencing (GSPGISGGGGGILDSDMAEP) indicates that both bands have the expected sequence, with 15 additional vector-derived residues upstream of the N-terminal sequence of BVR-A of MDAEP (underlined). The vector-derived amino acid sequence in the glycine linker region is LVPRGSPGISGGGGGILDSDMGRLE. This suggests that the heterogeneity, if indeed it occurs, is at the C-terminus, although there are no obvious thrombin sequences in this region. This heterogeneity is certainly only seen after thrombin cleavage because only one band is seen for the GST-BVR-A fusion protein on SDS/PAGE (60 kDa) and on isoelectric focusing (pI 6.92). The two BVR-A bands seen on SDS/PAGE were also seen when 2',5'-ADP-Sepharose-purified preparations of thrombin-cleaved BVR-A were subjected to isoelectric focusing, giving pI values of 6.29 and 6.44 (results not shown). Interestingly, on gel filtration the fusion protein runs as a dimer of 120 kDa. GSTs are known to exist as dimers [31] and in this instance a monomer (BVR-A) has been artificially 'dimerized' as a GST fusion protein. It seems unlikely that there are any subunit interactions in the dimer apart from those normally involved in stabilizing the native GST dimer, but further work is required to establish this. The initial-rate kinetics of BVR-A as the GST fusion protein (Figure 1a) and after thrombin cleavage (Figure 1b) are shown in the absence and the presence of BSA. There is clearly very little effect of the GST domain on the initial-rate kinetics of BVR-A: both forms exhibit substrate inhibition with similar kinetic parameters.

The presence or absence of the GST domain also has little effect on the pre-steady-state 'burst' kinetics of the recombinant rat kidney enzyme, which exhibits a pH dependence (Figure 2) similar to that previously reported for ox kidney BVR-A [24].

The effect of incubating BVR-A with BV and [4-³H]NADH labelled on the A face and the B face with GDH and ADH

respectively showed clearly that BVR-A is B-face-specific. The GDH and ADH reactions converted 75% and 71% of the NAD⁺ to NADH respectively; the subsequent BVR-A reactions showed 90% and 64% conversion of BV to BR. Under these conditions the incorporation of ³H into BR for the B-face reaction is essentially quantitative (51 317 d.p.m. compared with 2691 d.p.m. with NADH labelled at the A face). The radioactivity carried over in controls (i.e. no BVR-A added to the reaction mixture) was 3225 d.p.m. and 1463 d.p.m. for B-face and A-face controls respectively. A similar result has recently been observed with salmon liver BVR-A [23]. Although we have determined the stereospecificity of NADH oxidation by BVR-A it remains to be seen whether the reduction of BV to BR is also stereospecific.

Our ability to produce large quantities of BVR-A allows further structural work on the fusion protein (which does not exhibit the heterogeneity seen after thrombin cleavage) and provides a source of catalytically active BVR-A to act as a coupling enzyme for HO assays. Biliverdin-IX β reductase has been isolated [32] and its subsequent identity with flavin reductase [33] reported. Biliverdin-IX β reductase is clearly found in addition to BVR-A in liver cytosols [32,33] and, as biliverdin-IX β reductase is actually a 'non- α reductase' [32], most liver cytosols can probably reduce all four isomers of biliverdin. The common practice [34–37] of using liver cytosols as a source of BVR-A, to monitor specifically the conversion of the IX α isomer of biliverdin into bilirubin in assays for HO activity, will therefore also measure any HO activities that are not specific for the IX α isomer and should therefore be discouraged in favour of the use of homogeneous preparations of BVR-A.

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