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Activation of biliverdin-IX α reductase by inorganic phosphate and related anions.

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Abstract

The effect of pH on the initial rate kinetic behaviour of BVR-A exhibits an alkaline optimum with reduced nicotinamide adenine dinucleotide phosphate as cofactor but a neutral optimum with reduced nicotinamide adenine dinucleotide as cofactor. This has been described as “dual cofactor and dual pH dependent” behaviour however no mechanism has been described to explain this phenomenon. We present evidence that the apparent peak of activity at neutral pH seen with phosphate buffer and reduced nicotinamide adenine dinucleotide as cofactor is an anion-dependent activation where inorganic phosphate apparently mimicks the role played by the 2'-phosphate of reduced nicotinamide adenine dinucleotide phosphate in stabilising the interaction between reduced nicotinamide adenine dinucleotide and the enzyme. The enzymes from mouse, rat and human all exhibit this behaviour. This behaviour is not seen with biliverdin-IX α reductase from *Xenopus tropicalis* or the ancient cyanobacterial enzyme from *Synechocystis* PCC 6803 which, in addition to being refractory to

activation by inorganic phosphate, are also differentiated by an acid pH optimum with both pyridine nucleotides.

Introduction

The haem catabolic pathway is currently receiving attention in a number of fields with the observation that induction of haem oxygenase-1 (HO-1) is cytoprotective [1; 2]. Attempts to relate this to production of the immediate product biliverdin [3; 4] and/or subsequent reduction to bilirubin [5] or to a redox cycling mechanism between these two linear tetrapyrroles [6] are unresolved, however there is clear evidence that biliverdin has some unexpected and dramatic effects. Bach and co-workers have presented intriguing evidence that administration of biliverdin is cytoprotective for heart, colon and liver in animal transplantation studies [7; 8; 9]. In a striking report Bach and co-workers have shown that short term treatment (3 weeks) with biliverdin is sufficient to induce tolerance in a recipient to the donor heart for 120 days [9]. The introduction of an allogeneic third heart at 120 days was rejected whereas the introduction of a third syngeneic heart was accepted, clearly indicating tolerance.

Despite the current interest in the metabolism of linear tetrapyrroles, biliverdin-IX α reductase (BVR-A) has received relatively little attention. The reaction catalysed by BVR-A is illustrated in Scheme 1. BVR-A is a cytosolic monomer (34kDa), although the human enzyme migrates anomalously on SDS/PAGE with a mobility corresponding to a molecular weight reported variously as 46kDa [10] or 41kDa [11]. BVR-A catalyses the transfer of the B-face hydrogen [12] to a variety of verdin acceptors [13]. The structure of the rat enzyme [14] shows that the enzyme contains two domains, an N-terminal domain characteristic of a dinucleotide binding fold (Rossmann fold) and a C-terminal domain that is predominantly an antiparallel six-stranded beta-sheet. A structure for the rat enzyme with NADH bound has also been reported [15], however the adenine moiety folds back over the nicotinamide ring and this may, as the authors discuss, be an artefact of crystallisation where lysine-209 from a symmetry-related molecule precludes the adenine ring adopting the open structure predicted to exist in solution [14].

BVR-A is a small enzyme that exhibits complicated kinetic behaviour. The enzyme shows pronounced substrate inhibition (which may be modulated by intracellular biliverdin-binding proteins [16]) and also has two distinct pH optima depending on whether NADH or NADPH is the pyridine nucleotide. NADH is preferentially used between pH 6 and 7 while NADPH is the preferred cofactor between pH 8.5 to 8.7. This behaviour is seen with both native and recombinant human and rat BVR-A [17; 18; 19; 20], the ox kidney [21] and the pig spleen enzymes [22]. Interestingly one notable exception to this dual pH behaviour is the cyanobacterial BVR-A from *Synechocystis* PCC6803 which displays maximal activity at pH 5.8 when using either NADPH or NADH as cofactor [23]. During a re-investigation of this phenomenon (extended to include the recombinant enzymes from *Xenopus tropicalis* and mouse) we have discovered that the enzyme activity with NADH as cofactor is markedly activated by inorganic phosphate at neutral pH and present a novel mechanism to explain this observation.

Materials and Methods

Details on the purification of the salmon [24], rat [12], human [13] and *Synechocystis* [23] enzymes have been reported previously. The pBS152V *Synechocystis* BVR-A encoding plasmid was a kind gift from Dr. W. Schluchter, of the Department of Molecular and Cell Biology, University of California, Berkeley, California.

The mouse enzyme was cloned by RT/PCR using mRNA from Balb/c mouse liver. The specific primer used for the reverse transcriptase reaction was TGAAGCTTCCTCCTCACTGCTTCCGG and the primers for the PCR reaction were, forward primer: CCTCTAGAATGAGTACTGAGCCAA and reverse primer: CCAAGCTTCCTCCTCACTGCTTCC. The PCR product was digested with XbaI and HindIII to allow directional cloning into the maltose-binding protein fusion vector ppMAL. DNA sequencing of the murine BVR-A cDNA construct was carried out and compared with four EST sequences. The cDNA sequence of EST-1 (Accession Number AK002231.1) appears to have a stop codon 21 base

pairs after the stop codon of all other cDNA sequences aligned including that of the cloned product. An additional variation occurs in the codon for leucine-27 (TTG). In two of these sequences (accession numbers AK002231.1 and AK010847.1) and also the murine ppMAL-mBVR-A construct, the codon is as expected, however in 2 other EST's (accession numbers BC052146.1 and NM_026678.3), codon-27 reads (TCG) which codes for lysine at this position. The MBP-mBVR-A fusion protein was produced in *E.coli* strain BL21 transformed with ppMAL-mBVR-A. After induction with IPTG (0.2mM for 12 hours at 27°C) the cytosol was loaded onto an amylose affinity column (1.5 x 10cm) to bind the MBP/BVR-A fusion protein. This was washed extensively with 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7 and the fusion protein was then eluted with 10 mM maltose in 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7. The eluate was tested for BVR-A activity and the peak fractions pooled. From 1 litre of culture 20 mg MBP fusion was routinely obtained. The MBP was cleaved using PreScission™ Protease (Amersham Biosciences).

The enzyme from *Xenopus tropicalis* was cloned from the I.M.A.G.E cDNA clone (IMAGE:5308696) which was acquired from UK HGMP-RC, Hinxton, Cambridge CB10 1SB, UK [38]. Using the following forward (GGATGGATCCATGTTTGGCGCAGTAGT) and reverse (CCTCGAGCCATGGTTAATGCTCACATTTTC) primers an amplified product of the predicted size (~882 base pairs) was obtained and cut with BamHI and XhoI to allow directional cloning into pGEX-KG. The resulting plasmid (pGEX-KG-XeBVR-A) was transformed into *E.coli* BL21 and the GST-fusion protein isolated by affinity chromatography on glutathione-agarose (Chromatrin). Site directed mutagenesis of the rat enzyme was carried out using PCR as described by [25].

Initial rate kinetics were conducted by following the production of bilirubin by monitoring the increase in absorbance at 460 nm as described previously [13]. Assays were conducted at 30°C using a Unicam 8625 UV/Vis spectrophotometer connected to a Kipp and Zonen flatbed chart recorder. For routine assays the incubation mixture contained 0.1 M sodium phosphate buffer pH 7.2, 700 µM NADH, 10 µM biliverdin-IX α and the reaction was started by the addition of

enzyme. To determine the stability of BVR-A over the pH range studied the various enzymes were diluted 10 fold to a concentration of 0.1 mg/ml in 100 mM buffer at each pH. The diluted mixtures were incubated at 30°C for 10 minutes in the buffers indicated, after which time 10 μ l aliquots were assayed in 100mM Tris-HCl pH 8 (final volume 2ml), with a final concentration of 100 μ M NADPH and 10 μ M biliverdin. Initial rates are described as μ moles or nmoles produced per minute per mg enzyme. For some experiments, particularly the stopped-flow experiments, the rates are expressed as change in absorbance (ΔA) per minute. The stopped flow experiments were conducted using an Applied Photophysics π^* -180 spectrometer and Pro-K global analysis software.

The following buffers (100mM) were used to determine the effect of pH on the activity of BVR-A: sodium acetate (pH 5 - 6), sodium citrate (pH 5 - 6.5), sodium phosphate (pH 6.5 - 8) and Tris-HCl (pH 8 - 9). Assays for BVR-A activity were generally conducted in the absence of BSA. The addition of BSA gives an enhanced ΔA_{460} and maintains linear initial rates for longer, however it introduces complications when interpreting kinetic results since the total concentration of biliverdin is not identical with the free concentration as a significant fraction binds to BSA (or any other biliverdin binding protein [26; 16]). Biliverdin-IX α was prepared by oxidation of bilirubin-IX α [27].

Results and Discussion

The effects of pH on the stability of various BVR-As.

To determine the stability of the rat, murine and human recombinant BVR-A, the enzymes were pre-incubated in various buffers over the pH range 5 – 9. The human enzyme (Fig. 1a) lost no more than 5 – 10% of its activity even at the extremes of pH tested (pH5 and pH9). Under the conditions examined the enzyme was relatively dilute (0.1 mg/ml) and was pre-incubated for 10 minutes at the various pH values. In kinetic studies the reaction was generally initiated by the addition of enzyme and the initial rate measured from the initial 10 – 20 seconds of the reaction. It is unlikely therefore that any significant inactivation of

the human enzyme occurred in these initial rate experiments. The rat enzyme exhibited a similar tolerance to pH but was marginally less stable at alkaline values, losing 25% of its activity at pH 9 after a 10 minute pre-incubation (Fig. 1a). The mouse enzyme is considerably less stable at both acid and alkaline pH values and even at pH values between 6 and 8 loses 10 – 20% activity after 10 minutes incubation at 30°C (Fig. 1a). The relative instability of murine BVR-A at acid pH values prompted a further investigation to determine the kinetics of inactivation at pH 4 and 4.5. After 10 minutes pre-incubation at pH 4.5, only 10% of the activity remained (Fig. 1b). However 50% activity remains after 2 minutes pre-incubation at pH 4.5 (Fig. 1b) and extrapolation suggests that measurements up to 30 seconds at pH 4.5 will yield 90% of the original activity. It is not possible to extend this level of confidence to pH 4 where only 50% activity remains after 30 seconds pre-incubation (Fig. 1b). The degradation of NADH and NADPH under acid conditions has been reported in numerous studies see e.g. [28]. It is clear from these studies that the pH has a significant effect on the rate of NADPH and NADH degradation. At 30°C, the half life of NADPH was reported as just 38 minutes at pH 5 [28]. As a result stock solutions of NAD(P)H in water were prepared fresh each day and stored on ice.

The effect of pH on the activity of BVR-A

It is important to note that of all the BVR-A enzymes characterised to date the *Synechocystis PCC6803* and salmon enzymes are the only forms where activity has been surveyed over the entire pH range studied using both nucleotides [23; 29] and these, together with the eel (*Anguilla japonica*) enzyme [30], support significant activity at acid pH values.

A series of reactions were set up to determine the effects of pH on the activity of human, rat and *Xenopus* BVR-A and the results are presented in Figs 2a,b and c.

During the course of these experiments two interesting observations were made. The first is that the use of phosphate buffer substantially increased the rate for the NADH-dependent reaction over the pH range (from pH 6-8) used in these

experiments for the human and rat enzymes. The second point worth noting is that the NADPH-dependent activity of the human and rat enzymes is clearly not limited to the pH range of 7.5 to 9.

The activity of the *Xenopus* enzyme exhibited a pH optima of 4.75, which is similar to the behaviour of the *Synechocystis* enzyme [23]. The *Xenopus* enzyme is similar to the salmon enzyme [29] in that both are more active with NADH than NADPH.

The effects of various anions on the activity of human BVR-A

Fig. 3 shows the effect of increasing concentrations of phosphate buffer (pH 7) on the activity of human BVR-A with NADH as the cofactor. The data fits well to the Hill equation, as illustrated, however there are probably effects due to ionic strength in addition to the specific activation by phosphate anion. The activity in 5 mM phosphate is $0.04 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ and in 200mM phosphate this increases 37.5 fold to $1.5 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$. To determine whether this effect was specific to phosphate a range of concentrations of various buffers at pH 7 was tested. Assays were performed with $20 \mu\text{M}$ biliverdin and $700 \mu\text{M}$ NADH at 30°C in various concentrations of sodium phosphate (2, 5, 10, 25, 50, 75, 100 and 200 mM) and sodium carbonate, Tris-HCl and sodium citrate (all at 10, 50, 100 and 200 mM). The reaction was started by the addition of enzyme. The pH of each assay were tested before and after the assay to ensure that pH did not alter through the course of the assay especially when using salts at the limits of their buffering capacity.

The results of the experiment detailed in Fig. 3 clearly demonstrate that phosphate is a powerful activator when NADH is used as cofactor. There is a modest effect of ionic strength as shown by the increase in initial rates seen with increasing concentrations of sodium citrate, Tris-HCl and sodium carbonate up to the 200 mM. However the effect of phosphate on enzyme activity is particularly marked. Interestingly pyrophosphate also displayed an activatory effect, indeed the potency observed was greater than that obtained with phosphate (Fig. 4). It is also clear from Fig. 4 that the marked activation seen with both phosphate and

pyrophosphate is specific for NADH. There is only a very modest increase in activity seen with NADPH as cofactor over the same range. The basis for the activation however was unclear from these results. Phosphate may allow substrates to bind more effectively to the enzyme and/or enhance turnover.

The effects of phosphate on the NADH dependent activity of human BVR-A

A series of saturation curves were generated with NADH as the variable substrate (20 μ M to 3.2 mM) in 10, 50, 100, and 200mM sodium phosphate pH 7. Biliverdin-IX α at a final concentration of 20 μ M was used as substrate and the reaction was performed at 30°C. Data points were fitted to the Michaelis equation and the rectangular hyperbolas generated by these fits were used to obtain the apparent K_m and V_{max} (Table 1). Increasing phosphate from 10mM to 200mM resulted in a dramatic decrease in the apparent K_m for NADH (150-fold) with no significant change in the apparent V_{max} . Clearly inorganic phosphate promotes NADH binding without enhancing turnover.

The effect of phosphate on the burst rate constant of human BVR-A.

Stopped-flow studies on ox kidney [21] and rat [12] BVR-A shows a burst reaction which has been attributed to the slow release of bilirubin from an enzyme.NADP.bilirubin complex. The effect of phosphate on the pre-steady state kinetics was determined by monitoring the increase in absorbance at 460nm at 23°C. Human BVR-A (1.6 μ M) in 100mM sodium citrate pH 7.2 was rapidly mixed with 1.4 mM NADH, 14 μ M biliverdin-IX α in varying concentrations of sodium phosphate pH 7.2 (Fig. 5). The concentration of phosphate in the substrate syringe was varied to give final concentrations of 5, 10, 20, 50, 100 and 200 mM. The results of these stopped flow experiments were fitted to a curve of single exponential with slope. The steady state rate showed that at 23°C there is a pronounced activation by phosphate anion (8-fold compared with the rate in Tris-Cl) and the burst rate constants (k_b) obtained from these experiments are shown in the inset to Fig.5. There is a modest effect of phosphate concentration on the burst rate constant which essentially doubles between 0 and 50 mM phosphate from 0.15 s⁻¹ to 0.28s⁻¹.

The effect of pH on the activation by phosphate of human BVR-A using NADH as cofactor.

The effect of pH on the activity of human BVR-A with increasing concentrations of phosphate was also investigated (Fig. 6). The activity was measured in 100 mM sodium citrate over the pH range 5-7 with 7 μ M biliverdin-IX α and 100 μ M NADH as substrates. The concentrations of phosphate employed were 5, 10, 15, 20, 40, 60, 80, 100 mM. It is clear that at pH 5 the activation by phosphate anion is less potent than that seen at more neutral pH values. By plotting A_{50} (the concentration of activator giving 50% activation) against pH an apparent pK of approximately 4.9 can be identified (insert to Fig. 6). Deprotonation of this residue increases the affinity for phosphate anion 6-fold. A possible candidate for this pK can be tentatively identified as glutamate-75. The side chain of glu75 interacts with arg44 (one of the residues that interacts with inorganic phosphate in the rat structure) and is predicted to do so more strongly as the carboxylate anion. A weaker interaction as the protonated carboxyl may allow arg44 to adopt an alternative conformation that partially destabilises the arg44 interaction with phosphate.

Studies on phosphate activation of rat BVR-A: identification of a putative 2'-phosphate binding site

In an effort to further understand the nature of the activation of the NADH-dependent activity of human BVR-A by phosphate and related anions it was decided to analyse the two BVR-A structures then available ([14] [15]) for the rat enzyme. Although not commented on by the authors, rat BVR-A (Whitby *et al.*, 2002) [15] co-crystallised with three inorganic phosphate molecules [15]. Interestingly, one of these phosphates occupies a site which has been proposed as the binding pocket for the 2'-phospho ester of NADPH [14]. In light of this information it was decided to look at the residues involved in binding phosphate in more detail. As shown in Fig. 7, serine-43, arginine-44 and arginine-45 form an intricate lattice of ionic interactions and hydrogen bonds with inorganic phosphate.

Effect of phosphate concentration on the activity of wild type and “2'-phosphate pocket” mutants of rat BVR-A

Fig. 8 shows the effect of phosphate concentration on the activity of the rat enzyme (wild type) in Tris-HCl buffer. As with the human enzyme there is a marked activation by phosphate with an A_{50} value of 20 mM. Although all three of the desired mutants were created only the S43A and the R44A mutants were sufficiently stable to examine kinetically. The A_{50} for the S43 mutant increases significantly (Fig. 8) to 60 mM indicating that significant binding energy results from the interaction between serine 43 and the phosphate anion. A more dramatic result was seen with the R44A mutant which is not activated significantly by increasing concentrations of phosphate when NADH is used as cofactor (Fig.8). Mutation of these two residues in the “2'-phosphate pocket”, decreases (S43A), and abolishes (R44A) the ability of inorganic phosphate to activate the NADH-dependent reduction of biliverdin, clearly implicating this as the site involved in the phosphate-activation phenomenon. We have also confirmed an earlier report [14] that the R44A mutation equalises the apparent K_m for NADH and NADPH (data not shown) again implicating this as the binding site for the 2'-phosphoester of NADPH.

The conclusion from this study that NAD(P)H binds in an extended conformation and that inorganic phosphate binds to the 2'-phosphate pocket for NAD(P)H has recently been confirmed in the crystal structure of human BVR-A with NADPH which has been deposited in the Protein Data Bank by the Structural Genomics Consortium with code 2H63. Our model for the mechanism of activation of the NADH-dependent activity of BVR-A by inorganic phosphate therefore proposes that inorganic phosphate mimicks the 2'-phosphoester of NADPH. There are clearly questions remaining concerning the nature of this complex as an additional oxygen atom is present in the enzyme.NADH.Pi complex when compared with the enzyme.NADPH complex. Indeed the ability of pyrophosphate to activate suggests that at least some reorganisation of the enzyme structure is necessary to achieve the ternary complexes with anions and this will be the focus of further studies.

The hypothesis that NADH binding can be augmented by inorganic phosphate “mimicking” the 2'-phosphate of NADPH is not entirely novel as a similar conclusion has been reached by Christensen *et al.*, (2005) [31] for the Mg-dependent methylenetetrahydrofolate dehydrogenase-cyclohydrolase (NMDMC). These authors point out that NMDMC uses phosphate and magnesium to adapt an NADP site for NAD binding.

The effect of pH and biliverdin-IX α concentrations on the initial rate using NADPH as cofactor.

At 2 μ M biliverdin-IX α the human enzyme is active over the entire pH range studied (Fig. 9). As the substrate concentration is increased to 5 and 20 μ M the initial rate is significantly suppressed at neutral pH (due to pronounced substrate inhibition) which is far less severe under both acid and alkaline conditions (Fig. 9). This results in a shift in the apparent pH optimum from 8 to 8.5, depending on the biliverdin concentration used in the assay. The increased potency of substrate inhibition at neutral pH was also clearly evident in the time course of the reaction. An increase in rate during the time course of the reaction was observed as the substrate concentration decreased below substrate inhibitory levels. This was particularly apparent at neutral pH values, where substrate inhibition is pronounced, while at the acid and alkaline limbs, where substrate inhibition was not apparent more normal P(t) curves were observed (data not shown). It is still not clear why NADPH supports a significant rate at pH 8.5 to 9 but that NADH is unable to achieve this.

It should be noted that the approximate concentrations of pyridine nucleotides in rat liver have been reported as 120 μ M for NADPH, 35 μ M for NADP, 70 μ M for NADH, and 500 μ M for NAD [32; 33]. With the apparent K_m for NADPH of biliverdin reductases being in the low micro molar range, typically 1-10 μ M, the enzyme is most likely saturated with NADPH *in vivo*. In contrast, the apparent K_m for NADH is generally reported between 300 and 500 μ M, and with cytosolic concentrations of NADH generally around 70 μ M this is unlikely to be a physiologically significant substrate. In well oxygenated perfused rat liver the cytosolic concentration of inorganic phosphate has been reported as 5.3mM [34]

and therefore is not predicted to impinge on NADH-dependent activity. However inorganic phosphate in mitochondria can rise to 25mM and as this subcellular location has a higher NADH/NAD ratio and has recently been reported to contain BVR-A [35], inorganic phosphate may promote NADH-dependent BVR-A activity in mitochondria.

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Legends to Schemes

Scheme 1

BVR-A catalysed reduction of biliverdin-IX α .

Legends to Figures

Figure 1

(a) The effect of pH on the stability of BVR-A

The enzyme (2 μ g) was incubated at the pH indicated for 10 minutes and then diluted 100-fold into assay buffer, 100 mM Tris/HCl pH 8 with 100 μ M NADPH and 10 μ M biliverdin-IX α . The results are shown for the human (Δ), rat (\square) and mouse (\bullet) enzymes.

(b) The kinetics of inactivation of the mouse BVR-A at acid pH.

The enzyme (2 μ g) was incubated in 0.1M sodium acetate at pH 4 (\circ) and pH 4.5 (\bullet) for various times and then diluted 100-fold into assay buffer (100 mM Tris/HCl pH 8) with 100 μ M NADPH and 10 μ M biliverdin-IX α .

Figure 2 The effect of pH on the activity of BVR-A

The results are shown for the (a) human, (b) rat and (c) *Xenopus* enzymes. The enzyme was assayed at the pH indicated in the following buffers sodium citrate (\bullet), sodium phosphate (\blacktriangle), sodium acetate (\blacklozenge) and Tris/HCl (\blacksquare) using 700 μ M NADH as cofactor and sodium citrate (\circ), sodium phosphate (Δ), sodium acetate (\diamond) and Tris/HCl (\square) using 100 μ M NADPH as cofactor. A final concentration of 10 μ M biliverdin was used.

Figure 3 (a) The effect of various buffers on the NADH-dependent activity of human BVR-A

The activity of the human enzyme was assayed in the presence of 700 μ M NADH and 20 μ M biliverdin as substrate at 30°C in the various buffers at the concentrations indicated. The buffers used were sodium phosphate (Δ), sodium carbonate (\blacklozenge), Tris/HCl (\bullet), and sodium citrate (\square) at pH 7.

Figure 4 The effect of various anions on the NADH- and NADPH-dependent activity of human BVR-A.

The enzyme (5 μg) was assayed using 700 μM NADH as cofactor and 10 μM biliverdin as substrate at pH 7 in (\circ) sodium phosphate, and in (\square) sodium pyrophosphate. Also shown are the results when hBVR-A was assayed using 100 μM NADPH and 10 μM biliverdin as substrate in sodium phosphate (\bullet) pH 7 at the concentrations indicated.

Figure 5 The effect of phosphate on the pre-steady burst of hBVR-A

The activity of the human enzyme (0.8 μM) was assayed in the presence of 700 μM NADH and 7 μM biliverdin with increasing concentrations of phosphate (0, 5, 10, 50, 100 and 200 mM) in 50 mM Tris pH 7.2.

Figure 6 Effect of pH on the phosphate-dependent activation of hBVR-A

The activity of human BVR-A was measured in 100mM sodium citrate over the pH range 5-7 (\bullet pH 5, \circ pH 5.5, \square pH 6, \blacksquare pH 6.5, and \diamond pH 7) with 7 μM biliverdin-IX α and 700 μM NADH. Phosphate concentration was increased from 0 to 100mM phosphate at each pH measured. Inset is a plot of A_{50} against pH fitted to a single pK.

Figure 7 The inorganic phosphate pocket of rBVR-A

The coordinates used for this Figure are taken from the crystal structure of the rat enzyme reported by Whitby et al (2002) [15]. The structure was downloaded from the Protein Data Bank [36], and graphics generated using the pyMol program published by DeLano Scientific [37]. Red dots represent water molecules. A surface diagram of the residues is shown in (a). The phosphate pocket is clearly defined with a large distribution of positive charges contributed by R 44 and R45 which lie along the walls of this pocket (coloured blue) and are seen to co-ordinate with the phosphate anion of the crystal structure (shown in b for clarity). Figure 7 c highlights the salt bridge formation between R44 and E75. This interaction may contribute to the stabilisation of the loop between B-sheets β 2 and β 3, and the α D helix in the rat crystal structure. Residues S43, R44 and R45

were targeted for mutation, in an attempt to determine their effects on NADH dependent phosphate activation.

Figure 8 The effect of phosphate on the activity of the wild type, S43A and R44A mutant rBVR-A

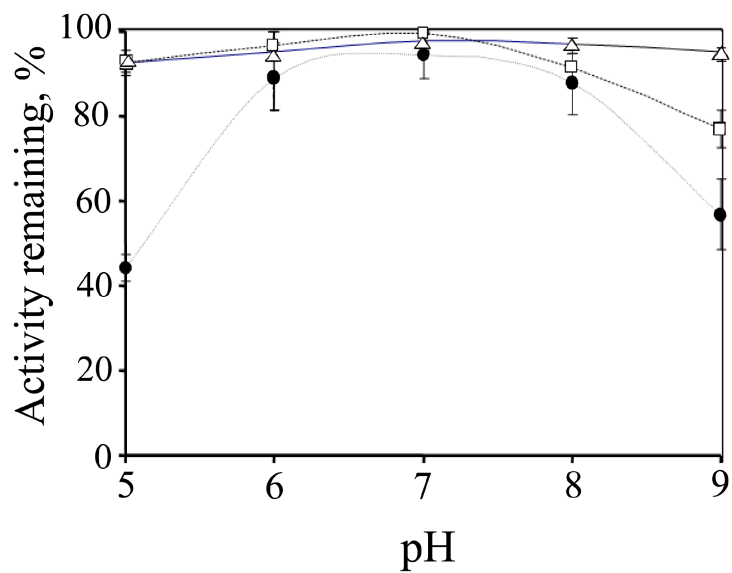
The activity of wild type rat BVR-A (\square), R45A BVR-A (\circ), and S44A BVR-A (\diamond), was measured in 50 mM Tris/HCl at pH 7.2 with 700 μ M NADH and 10 μ M biliverdin-IX α as substrate over the phosphate concentrations range (0 to 250 mM).

Figure 9 The effect of biliverdin concentration on the pH dependence of hBVRA activity.

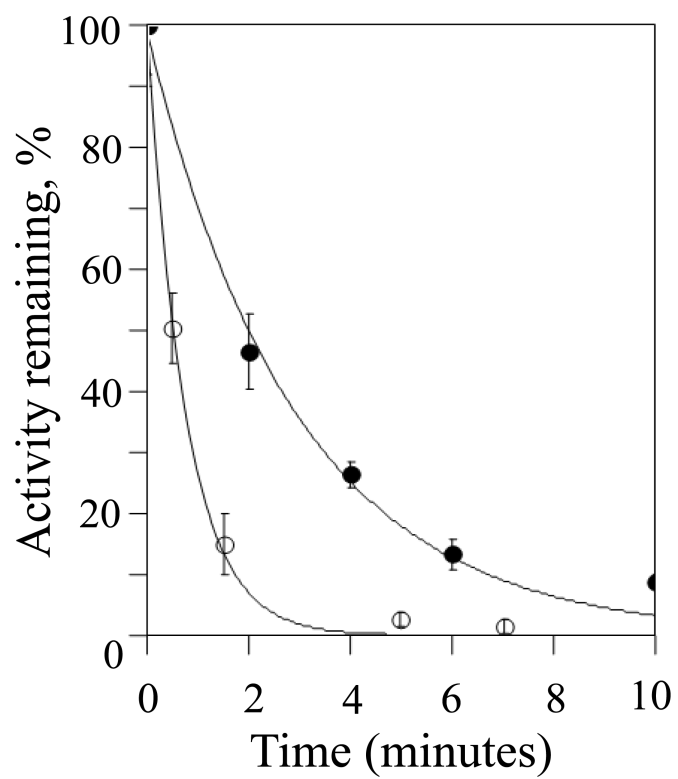
The effect of biliverdin concentration on the pH dependence of hBVRA activity was investigated. The reaction was monitored at 460 nm at 30°C, and contained a final concentration of 100 μ M NADPH with 2, 5 and 20 μ M biliverdin-IX α over the pH range 4.5-8.5.

Table 1 The apparent K_M and V_{max} of human BVR-A for NADH with increasing concentrations of sodium phosphate.

Data points in Fig. 6 were fitted to the Michaelis equation and the rectangular hyperbolas generated by these fits were used to obtain the apparent K_m and V_{max} .

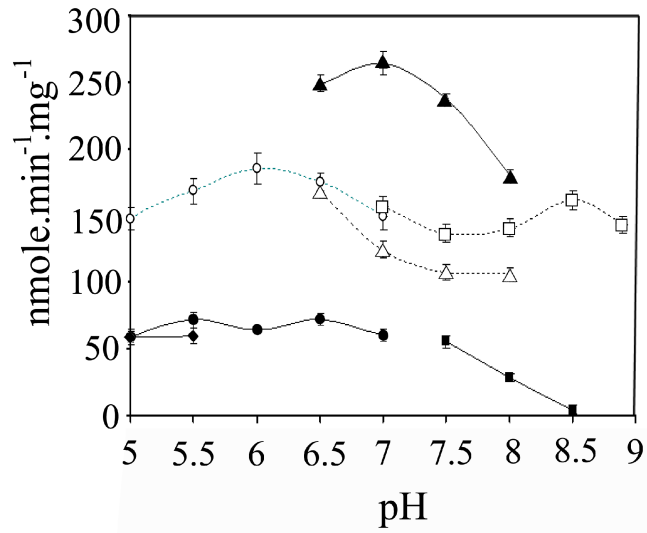


(a)

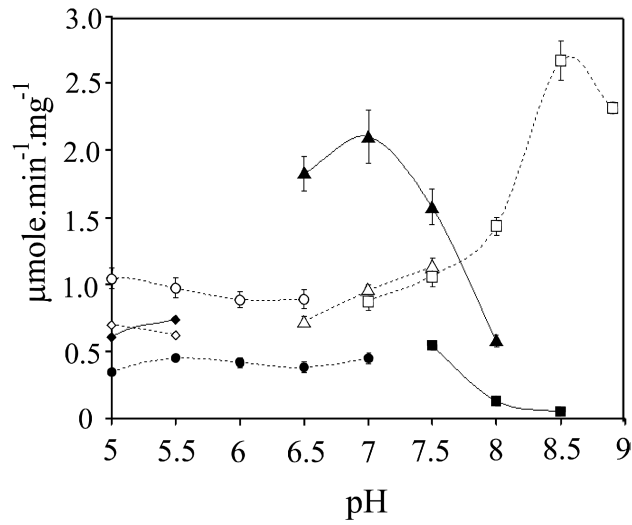


(b)

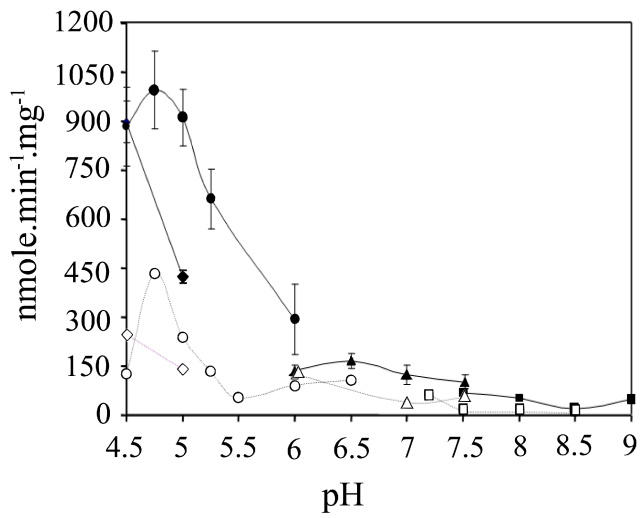
Figure 1



(a)



(b)



(c)

Figure 2

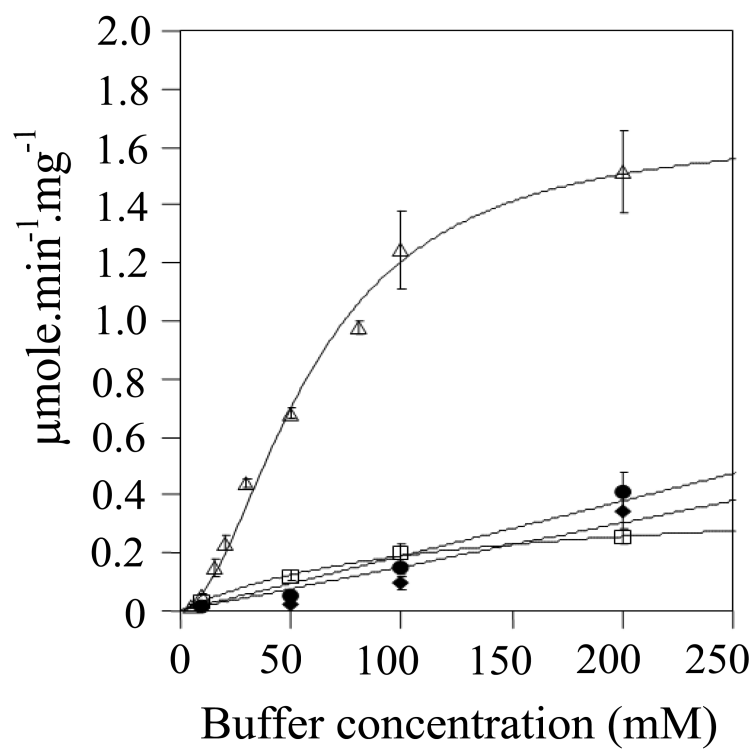


Figure 3

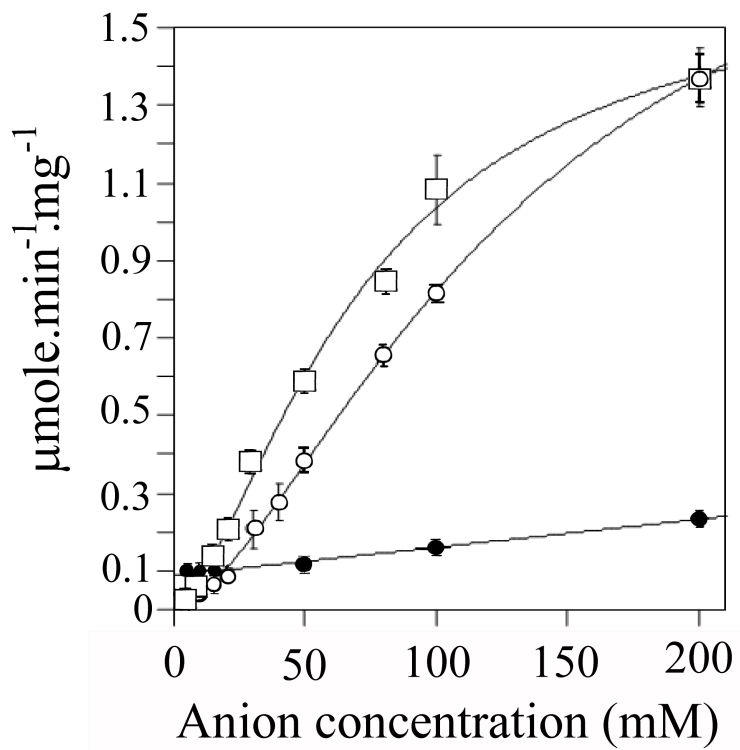


Figure 4

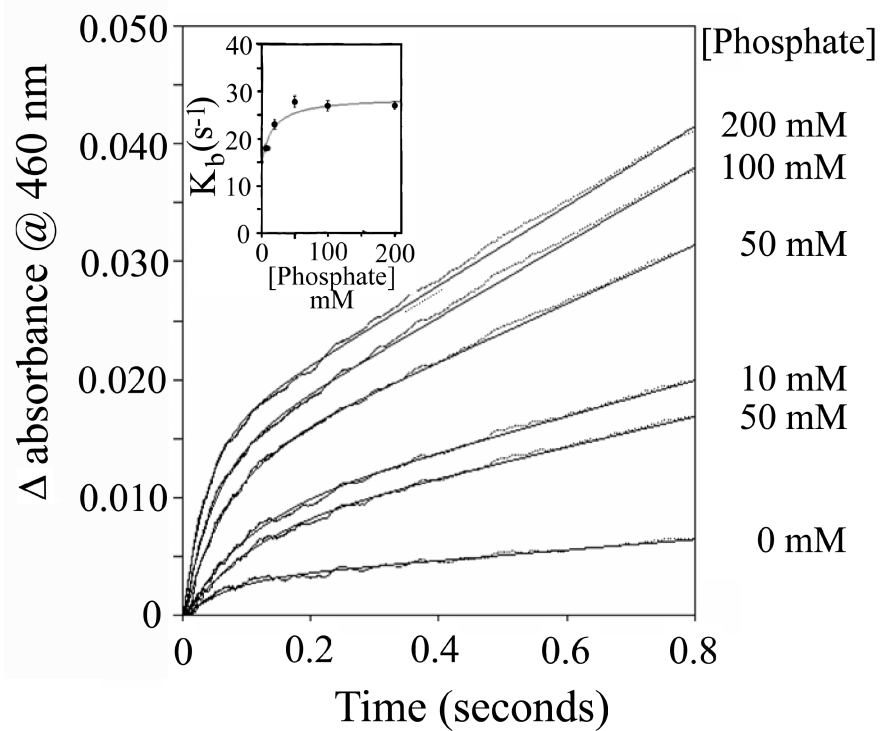


Figure 5

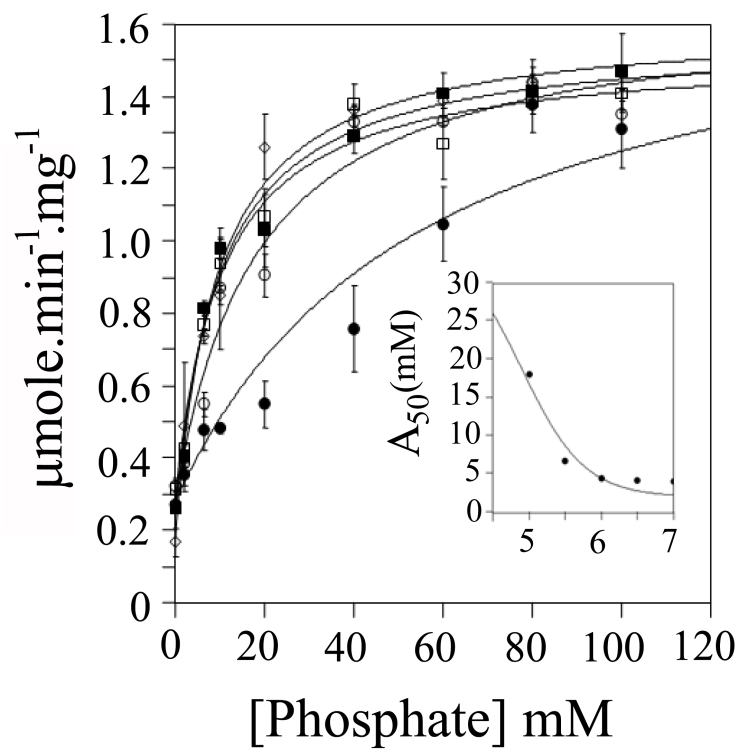
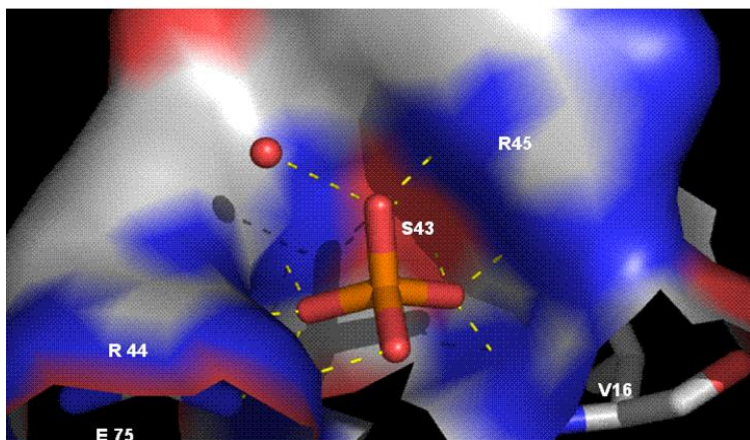
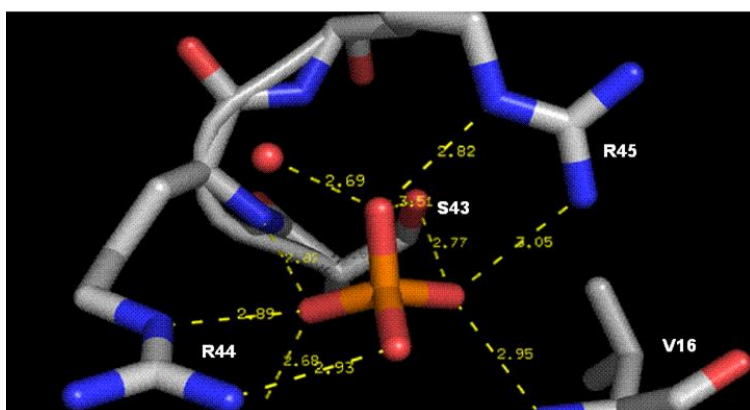


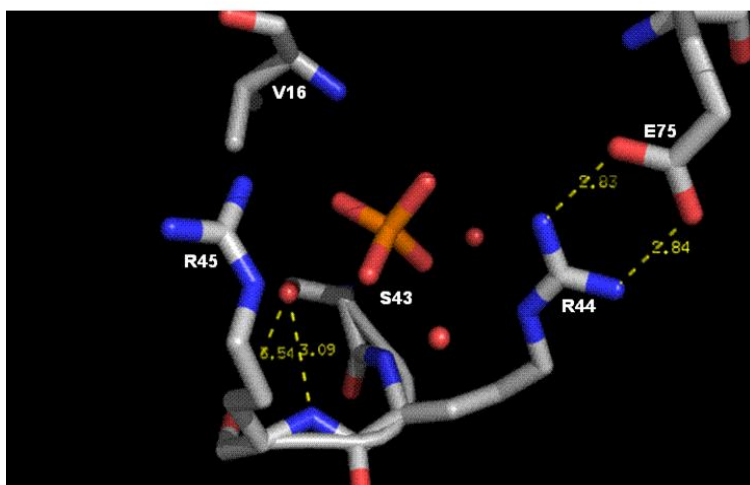
Figure 6



(a)



(b)



(c)

Figure 7

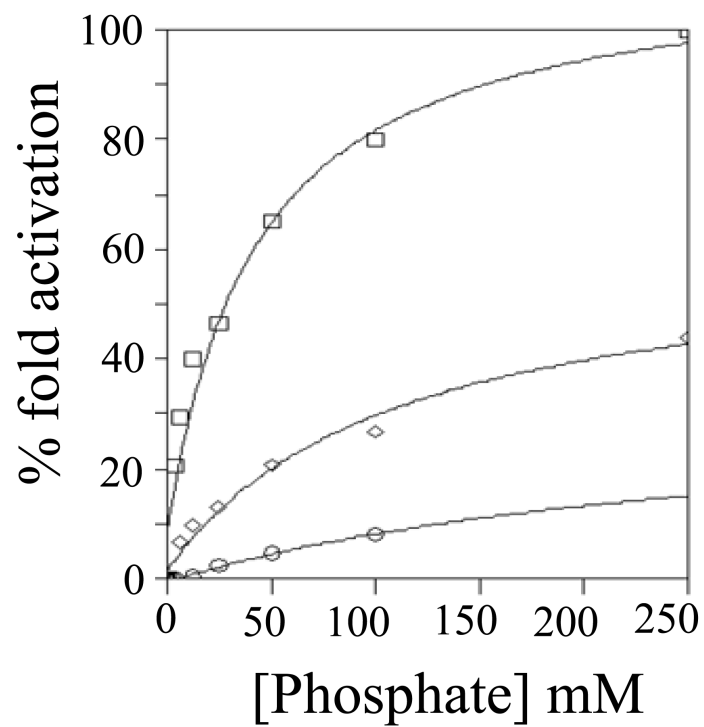


Figure 8

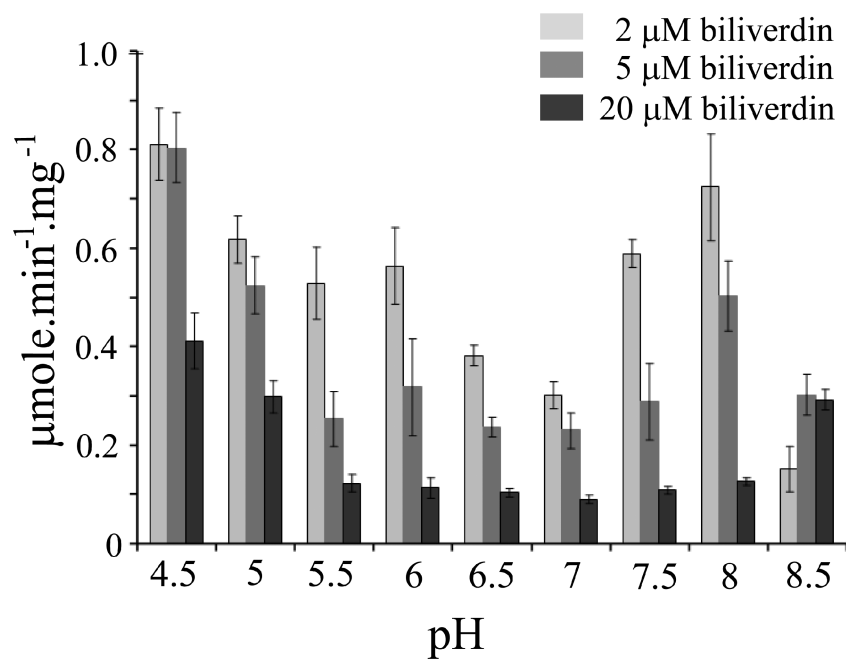
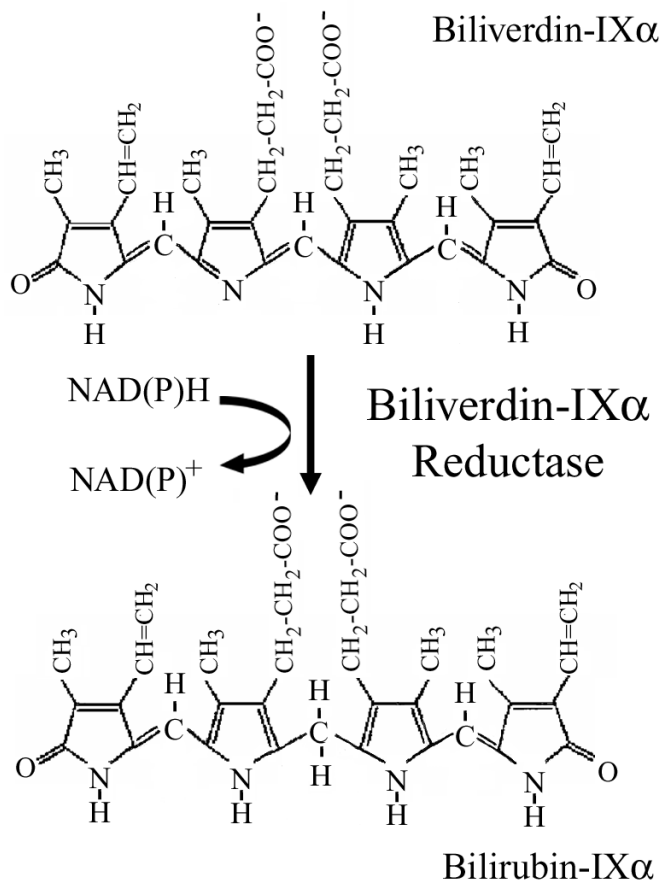


Figure 9

[Phosphate] (mM)	appKm ^{NADH} (mM)	appVmax ($\mu\text{mole}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)
10	39.20 +/- 69	2.00 +/- 3.77
50	3.05 +/- 0.74	2.03 +/- 0.29
100	0.79 +/- 0.18	2.00 +/- 0.21
200	0.22 +/- 0.02	2.12 +/- 0.07

Table 1



Scheme 1