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Activation of human biliverdin-IXα reductase by urea: Generation of kinetically distinct forms during the unfolding pathway

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ABSTRACT

Activation of enzymes by low concentrations of denaturants has been reported for a limited number of enzymes including lipocalin-type prostaglandin D synthase (L-PGDS) and adenylate kinase. During unfolding studies on human biliverdin-IXα reductase it was discovered that the enzyme is activated at low concentrations of urea. Under standard assay conditions the native enzyme displays pronounced substrate inhibition with biliverdin as variable substrate; however in the presence of 3 M urea, the substrate inhibition is abolished and the enzyme exhibits Michaelian kinetics. When the initial rate kinetics with NADPH as variable substrate are conducted in 3 M urea, the V_{max} is increased 11-fold to 1.8 μmol/min/mg and the apparent K_m for biliverdin increases from 1 to 3 μM. We report the existence of two kinetically distinct folded intermediates between the native and unfolded forms. When the period of incubation with urea was varied prior to measuring enzyme activity, the apparent V_{max} was shown to decay to half that seen at zero time with a half life of 5.8 minutes, while the apparent K_m for NADPH remains constant at approximately 5 μM. With NADH as cofactor the half life of the activated (A) form was 2.9 minutes, and this form decays in 3 M urea to a less active (LA) form. The apparent K_m for NADH increases from 0.33 mM to 2 mM for the A and LA forms. These kinetically distinct species are reminiscent of the activity-enhanced and inactive forms of L-PGDS observed in the presence of urea and guanidine hydrochloride.

1. Introduction

Heme oxygenase (HO) catalyses the conversion of free heme to the linear tetrapyrrole biliverdin (BV) with the concurrent release of carbon monoxide [1]. Biliverdin is subsequently converted to bilirubin by biliverdin-IXα reductase (BVR-A), a monomeric cytosolic pyridine nucleotide-dependent oxidoreductase. The specificity of BVR-A is limited to various isomers of biliverdin in contrast to biliverdin-IXβ reductase (BVR-B) which, in addition to various isomers of biliverdin, also reduces flavins, PQQ and ferric ion [2]. The product of the reaction, bilirubin-IXα, is conjugated with glucuronic acid in a reaction catalysed by UDP-glucuronosyltransferase (UGT1A1) and subsequently excreted into bile by mrp2 [3]. While this pathway was originally considered purely catabolic, several lines of evidence now suggest that the stress-inducible HO isoform, HO-1, is a major cytoprotectant. In agreement with this, induction of HO-1 and its metabolites protects against a large spectrum of diseases as demonstrated in animal models of colitis, psoriasis and multiple sclerosis [4–7]. There has been much discussion as to whether this is "hide the haem" or whether the HO-1 reaction produces a protective intermediate. Biliverdin, bilirubin and carbon monoxide have all been proposed as candidates in this regard [8,9]. In the case of biliverdin and bilirubin, this has been attributed not only to their antioxidant properties but also to emerging immunomodulatory properties which include direct effects on cells of the innate and adaptive immune system [10].

In addition to studies on the physiological role of BVR-A, it has been a useful model enzyme being one of a small number of monomeric oxidoreductases. The enzyme exhibits pronounced substrate inhibition with biliverdin as the variable substrate and this can be modulated by biliverdin binding proteins such as albumin and various members of the glutathione *S*-transferase family [11]. Earlier studies also demonstrated that it has two distinct pH optima depending on whether NADH or NADPH is the nicotinamide nucleotide. This was originally described as dual cofactor/dual pH-dependent behaviour with NADH preferentially used between pH 6 and 7, whereas NADPH is the preferred cofactor between pH 8.5 and 8.7. We have recently shown that the apparent peak of activity observed at neutral pH with phosphate buffer and NADH as cofactor is an anion dependent activation, where inorganic phosphate apparently mimics the role played by the 2′-phosphate of NADPH in stabilising the interaction between NADH and the enzyme [12]. While studying hBVR-A as a model for protein unfolding, we observed that low concentrations of urea activated the enzyme with higher concentrations showing concomitant changes in

Abbreviations: hBVR-A, human biliverdin-IXα reductase; PQQ, pyrroloquinoline quinone; UGT1A1, bilirubin UDP-glucuronosyltransferase 1; Mrp2, multidrug resistance protein 2; HO-1, haem oxygenase 1; ROS, reactive oxygen species; QM/MM, quantum mechanics/molecular-mechanics; ΔG_u, the free energy of unfolding

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protein fluorescence and enzyme inactivation. The mechanism of activation in this case is quite distinct to that seen with inorganic phosphate and is observed with both NADH and NADPH as cofactor. Here we report the detection of an intermediate on the unfolding pathway that is kinetically distinct to the native enzyme. Interestingly this unstable intermediate form is not subject to the characteristic potent substrate inhibition displayed by the native enzyme [11].

2. Materials and methods

2.1. Expression and purification of human BVR-A

Human BVR-A was expressed and purified as described previously [2].

2.2. Intrinsic fluorescence and protein folding studies

Protein unfolding was monitored by measuring changes in protein fluorescence as the concentration of denaturant was increased [13]. For estimates of the free energy change for the folding/unfolding reaction a two state mechanism was assumed, and the equilibrium constant K_U was used in the calculation of the free energy of unfolding $(\Delta G_u) = -RT\ln K_U$, where R is the gas constant (1.987 cal/deg/mol) and T is the absolute temperature. The free energy of unfolding (ΔG_u) was then plotted against the concentration of denaturant and extrapolated to calculate ΔG_u (H_2O) [13]. For denaturation experiments, urea and guanidine hydrochloride were prepared in 100 mM sodium phosphate, pH 7.2. All reactions were carried out at 25 °C. In experiments defined as "zero preincubation," the measurement of protein fluorescence and enzyme activity were measured as soon as possible (within seconds).

Table 1
The effect of 3 M urea on initial rate kinetic parameters and dissociation constants for hBVR-A. Kinetic parameters were determined from the data shown in Figs. 1 and 3.

	− Urea		+ Urea	
	K_m (μM)	V_{max} (μmol/min/mg)	K_m (μM)	V_{max} (μmol/min/mg)
NADPH	1	0.165	3	1.8
NADH	330	1.2	2000	6
	K_i (μM)	K_D (μM)	K_i (μM)	K_D (μM)
NADPH	–	1.92	–	6.77
$NADP^+$	1.6	1.85	0.35	5.23
NAD^+	300	–	1700	–
Biliverdin	28	–	147	–

2.3. Dissociation constants for pyridine nucleotides binding to human BVR-A

Dissociation constants for both NADPH and $NADP^+$ binding to hBVR-A were determined by monitoring the quenching of protein fluorescence in the presence and absence of 3 M urea. The fluorescence intensity F_{meas} was determined after each addition of nucleotide and subsequently corrected (F_{corr}) for the "inner filter effect" contributed by the increasing nucleotide concentrations. The corrected fluorescence was calculated according to the equation as described by Levine [14]. The extinction coefficients for NADPH and $NADP^+$ were calculated by measuring the absorbance of each cofactor at 295 nm (and 340 nm for the reduced form) over a series of concentrations where Beer's Law is obeyed. The dissociation constants were derived from titration plots of the percentage of total Δf_{corr} against nucleotide concentration.

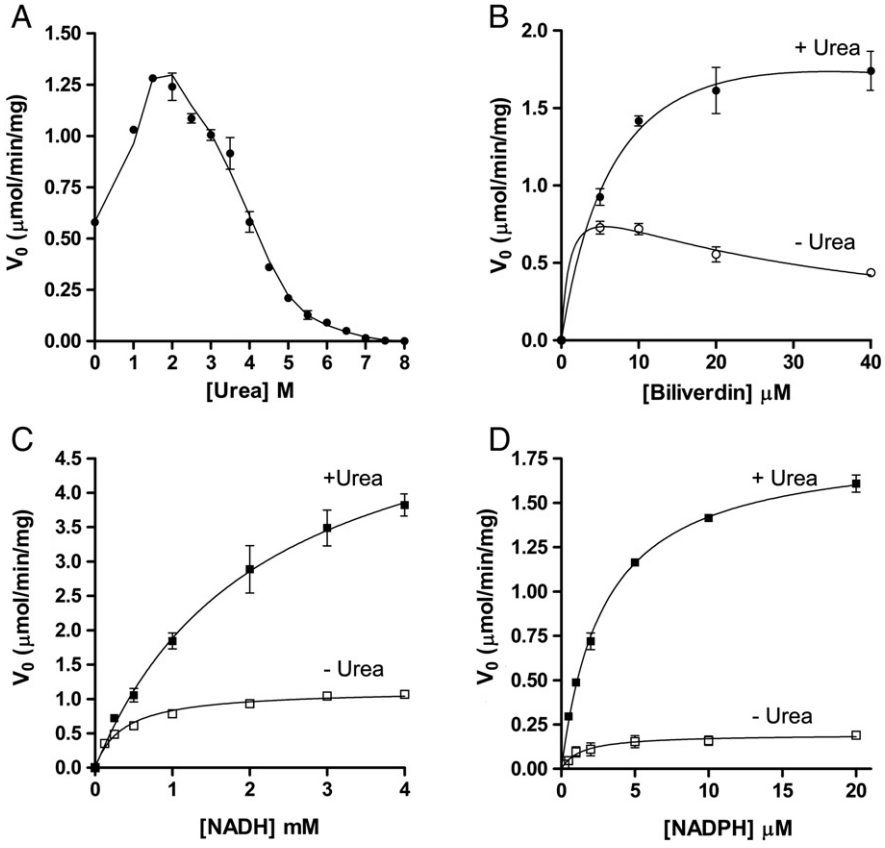

Fig. 1. The effect of urea on hBVR-A activity. Effect of increasing urea concentration on hBVRA activity (A). Bilirubin production was monitored at 460 nm over the range of urea concentrations indicated. The assay mix contained 705 μM NADH, 20 μM biliverdin and recombinant human BVR-A at 0.135 mg/ml. The effect of 3 M urea on the initial rate kinetics of BVR-A with biliverdin as variable substrate (B). Saturation curves with biliverdin as variable substrate were generated in the presence (●) and absence (○) of 3 M urea using 100 μM NADPH as cofactor in 100 mM sodium phosphate pH 7.2. The fit in the absence of urea (○) is to the equation for partial substrate inhibition as described in Section 2.4. The determination of apparent K_m and V_{max} values for NADH (C) and NADPH (D) in the presence (■) and absence (□) of 3 M urea is also shown.