RESEARCH COMMUNICATION

Evidence that biliverdin-IXβ reductase and flavin reductase are identical

Fiona SHALLOE, Gordon ELLIOTT, Orla ENNIS and Timothy J. MANTLE*

Department of Biochemistry, Trinity College, Dublin 2, Ireland

A search of the database shows that human biliverdin-IXβ reductase and flavin reductase are identical. We have isolated flavin reductase from bovine erythrocytes and show that the activity co-elutes with biliverdin-IXβ reductase. Preparations of the enzyme that are electrophoretically homogeneous exhibit both flavin reductase and biliverdin-IXβ reductase activities; however, they are not capable of catalysing the reduction of biliverdin-IXα. Although there is little obvious sequence identity between biliverdin-IXα reductase (BVR-A) and biliverdin-IXβ reductase (BVR-B), they do show weak immunological cross-reactivity. Both enzymes bind to 2′,5′-ADP–Sepharose.

INTRODUCTION

Haem catabolism in adult humans proceeds predominantly through the IXα isomers of biliverdin and bilirubin, reflecting the positional specificities of haem oxygenase (both forms I and II) and biliverdin-IXα reductase [1]. There has been considerable interest recently that this pathway (the haem catabolon) may produce a physiologically important antioxidant in bilirubin-IXα. Ames and co-workers [2] first suggested this on the basis of *in vitro* experiments, and the hypothesis is strengthened by the fact that haem oxygenase (HO-1), the first enzyme in the haem catabolon, is now known to be an important stress protein [3]. The hypothesis that bilirubin-IXα may have some beneficial effect has received further support from the observation that low serum bilirubin is associated with an increased risk of coronary artery disease [4]. Bilirubin-IXα inhibits the oxidation of human low-density lipoprotein better than does Trolox [5]. It has been suggested that this is mediated by interaction with low-density lipoprotein's α-tocopherol [6]. We have described the purification of biliverdin-IXα reductase (BVR-A) from ox kidney [7] and presented details of the steady-state and pre-steady-state mechanism [8,9]. The kinetics of this enzyme are complicated by pronounced substrate inhibition [10], and we have shown that this is modulated by intracellular biliverdin-IXα binding proteins [11].

Whereas the foregoing applies to adult physiology, it appears that the position is less clear cut in the foetus. Nakajima and co-workers [12] have shown that human foetal bile at 20 weeks of gestation contains 87% of the IXβ isomer of bilirubin. They have purified an apparently novel biliverdin-IXβ reductase, although the origin of the biliverdin-IXβ is not yet clear. This protein (isoenzyme I) has a molecular mass of 21 kDa [12,13], with no apparent sequence relatedness to human biliverdin-IXα reductase (39–42 kDa), which has recently been reported to be a zinc protein [1,12–14].

Human flavin reductase (FR) catalyses the NADPH-dependent reduction of FMN and Methylene Blue and, in the presence of redox couplers, the reduction of methaemoglobin. Although it exhibits methaemoglobin reductase activity *in vitro*, it is believed to contribute very little to methaemoglobin reduction under normal conditions [15]. However, the catalysis of methaemoglobin reduction by FR in the presence of Methylene Blue or riboflavin is the basis for the use of these compounds in congenital and toxic methaemoglobinaemia. FR has been shown to be identical with a major red-cell protein of unknown function termed 'green haem-binding' protein, found in erythrocytes and liver [16]. As noted by these workers, a physiological function for FR has not been clear. We now propose that one function is the reduction of biliverdin-IXβ.

We have isolated FR from bovine red cells using a novel affinity step and show that it co-elutes with biliverdin-IXβ reductase (BVR-B) activity. The purified protein, homogeneous by the criteria of SDS/PAGE, expresses both activities, although it is not capable of catalysing the reduction of biliverdin-IXα.

MATERIALS AND METHODS

Materials

2′,5′-ADP–Sepharose was obtained from Pharmacia. Biliverdin-IXα was synthesized as described previously [8], and biliverdin-IXβ was synthesized by ascorbate-coupled oxidation of haemoglobin as described previously [18]. This protocol produces a mixture of biliverdin-IXα (65%) and -IXβ (35%), which we have used directly, monitoring the production of bilirubin-IXβ at 450 nm [12]. Biliverdin-IXα reductase was expressed as a glutathione S-transferase fusion protein (60 kDa) as described previously [19]. The affinity-purified material was cleaved by digestion with thrombin, and the biliverdin-IXα reductase (34 kDa) was isolated by affinity chromatography on 2′,5′-ADP–Sepharose [7].

Purification of FR from bovine red cells

This was achieved using a modification of the methods described previously [20]. Blood was obtained from a local abattoir, using citrate as an anticoagulant. Packed red cells were obtained by centrifugation at 2000 ***g*** for 10 min at 4 °C. They were washed twice with 2 vol. of ice-cold saline (0.9% NaCl) and then lysed by the addition of 3 vol. of ice-cold distilled water. The stroma was removed by centrifugation at 13000 ***g*** for 1 h, and the red

Abbreviation used: FR, flavin reductase.

* To whom correspondence should be addressed.

supernatant was fractionated by $(NH_4)_2SO_4$ precipitation between 40 and 70 % saturation. This material was dialysed against 3 mM sodium phosphate, pH 7.2, and loaded on to a column of DEAE-cellulose equilibrated with the same buffer. The enzyme was eluted using a salt gradient (3–100 mM sodium phosphate, pH 7.2; 2 × 750 ml). Fractions displaying FR activity (see below) were pooled, concentrated using an Amicon ultrafiltration device and gel filtered on Ultrogel AcA 54. This material was essentially homogeneous; however, there was a minor contaminant (10 kDa). This was removed by adsorbing the enzyme to 2′,5′-ADP–Sepharose and eluting with 1 M NaCl in 10 mM sodium phosphate, pH 7.2.

SDS/PAGE

SDS/PAGE was carried out by the method of Laemmli [21], using 15 % gels.

Protein sequencing

A purifed sample of FR/biliverdin-IXβ reductase was sequenced (N-terminus; 15 residues) by the Welmet Protein Sequencing Service at the University of Edinburgh.

Enzyme assays

FR was assayed by the method of Yubisui et al. [22], and biliverdin-IXα reductase activity was measured by following the appearance of bilirubin-IXα at 460 nm [8]. Biliverdin-IXβ reductase activity was monitored by following the increase of bilirubin-IXβ at 450 nm [12].

RESULTS AND DISCUSSION

On aligning the sequences of human FR [22a] and biliverdin-IXβ reductase [13], it is clear that these two proteins are identical (Figure 1), with the exception that the initiation methionine and C-terminal glutamine are not identified in the protein sequence reported by Yamaguchi et al. [13]. To obtain experimental evidence for this conclusion, we purified bovine FR and monitored biliverdin-IXβ reductase activity during the preparation. The procedure that we describe for purifying FR yields 6 mg (37-fold purification) from 1 litre of whole blood and is relatively straightforward. The specific activity (240 nmol/min per mg of protein) is comparable with that described for the related human enzyme [22]. The protein is a monomer (23 kDa), exhibits a pI of 6.8 in 8 M urea and is identical (Figure 2) over the first 15 residues with the N-terminal sequence for bovine FR reported previously [23]. It is clear by these criteria that the protein we have isolated is identical with the FR purified by Hultquist's group [23]. Figure 3 shows the results of gel filtration of partially purified FR (after the DEAE-cellulose step). FR, biliverdin-IXα reductase and biliverdin-IXβ reductase activities were monitored. There was no biliverdin-IXα reductase activity; however, FR and biliverdin-IXβ reductase activities clearly co-eluted. This result is entirely consistent with our conclusion, based on an analysis of the sequence database, which indicates that these two proteins are identical. It should be noted that our use of a mixture of IXα and IXβ isomers of biliverdin is justified by the total lack of activity of FR/biliverdin-IXβ reductase with the IXα isomer (the present work and [12]). We have also shown that on completion of the biliverdin-IXβ reductase reaction the biliverdin-IXα remaining from the mixture is completely reduced to form bilirubin-IXα by adding biliverdin-IXα reductase. Preparations of bovine erythrocyte FR, homogeneous by the criteria of SDS/PAGE and giving unambiguous N-terminal sequence data, exhibit biliverdin-IXβ reductase activity of 160 nmol/min per mg of protein compared with the value of 331 nmol/min per mg of protein reported for human liver biliverdin-IXβ reductase [12]. In preliminary experiments (results not shown) we have obtained evidence for limited immunological cross-reactivity between biliverdin-IXα reductase (BVR-A) and biliverdin-IXβ

```
               10         20         30         40         50         60
hFR     MAVKKIAIFGATGQTGLTTLAQAVQAGYEVTVLVRDSSRLPSEGPRPAHVVVGDVLQAAD
        :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
hBVR-B  AVKKIAIFGATGQTGLTTLAQAVQAGYEVTVLVRDSSRLPSEGPRPAHVVVGDVLQAAD
               10         20         30         40         50

               70         80         90        100        110        120
hFR     VDKTVAGQDAVIVLLGTRNDLSPTTVMSEGARNIVAAMKAHGVDKVVACTSAFLLWDPTK
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
hBVR-B VDKTVAGQDAVIVLLGTRNDLSPTTVMSEGARNIVAAMKAHGVDKVVACTSAFLLWDPTK
      60         70         80         90        100        110

               130        140        150        160        170        180
hFR      VPPRLQAVTDDHIRMHKVLRESGLKYVAVMPPHIGDQPLTGAYTVTLDGRGPSRVISKHD
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
h-BVR-B VPPRLQAVTDDHIRMHKVLRESGLKYVAVMPPHIGDQPLTGAYTVTLDGRGPSRVISKHD
      120        130        140        150        160        170

              190        200
hFR     LGHFMLRCLTTDEYDGHSTYPSHQYQ
        :::::::::::::::::::::::::
hBVR-B LGHFMLRCLTTDEYDGHSTYPSHQY
     180        190        200
```

Figure 1 Amino acid sequences of human FR (hFR) and human BVR-B (hBVR-B)

The aligned protein sequences of human FR [22a] and human biliverdin-IXβ reductase [13] are shown.

```
hFR       MAVKKIAIFGATGQTG
bFR[23]    VVKKIALFGATGNVT
bFR*       VVKKIALFGATGNVT
             L          G/V
```

Figure 2 N-terminal amino acid sequences of human FR (hFR) and bovine FR (bFR)

The N-terminal amino acid sequences of human FR [22a] and bovine FR determined in [23] and the present work (*) (the fourth and fifteenth cycles also produced the Land G/V residues) are indicated.

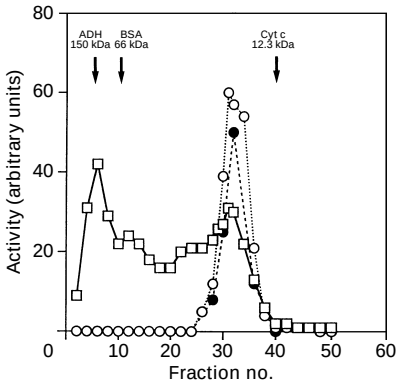

Figure 3 Gel filtration on Ultrogel AcA 54

A sample of partially purified FR (ex-DEAE-cellulose; 45 mg) was gel filtered, and activity was monitored for FR (○) and biliverdin-IXβ reductase (●). No fraction exhibited biliverdin-IXα reductase activity. The activity is represented as arbitrary units, which were $10^3 \times \Delta A_{340}$/min for FR and $(2 \times 10^2) \times \Delta A_{450}$/min for biliverdin-IX$\beta$ reductase. The open squares are absorbance at 280 nm. Abbreviations used: ADH, alcohol dehydrogenase; Cyt c, cytochrome *c*.

reductase (BVR-B). Attempts to align the sequences for rat BVR-A and human BVR-B reveal little similarity, although there are some identities at the N-terminus. Both enzymes bind efficiently to 2′,5′-ADP–Sepharose, indicating that similarities may exist in this domain. The capability of BVR-B to transfer reducing equivalents from NADPH to FMN and to biliverdin-IXβ raises the question of the number of independent substrate-binding sites that exist on this protein. Further work is required to resolve a number of structural and mechanistic questions that these observations raise.

Additionally the source of biliverdin-IXβ *in vivo* needs to be addressed. Xu et al. [16] have reported that FR/BVR-B is present in liver and other tissues, and we have shown that FR/BVR-B is present in isolated rat liver hepatocytes (F. Shalloe and M. Woods, unpublished work). This raises the intriguing possibility that a novel haem oxygenase may exist with a positional specificity for the β-methine bridge. An additional possible source may be foetal haemoglobin. Adult haemoglobin, on coupled oxidation with ascorbate, produces a mixture of IXα and IXβ isomers of biliverdin; however, it is not yet clear what isomers of biliverdin are produced by coupled oxidation of foetal haemoglobin.

This work was supported by the Health Research Board and BioResearch Ireland.

REFERENCES

1 Maines, M. D., Polevoda, B. V., Huang, T.-J. and McCoubrey, W. K. (1996) Eur. J. Biochem. **235**, 372–381

2 Stocker, R., Yamamoto, Y., McDonagh, A. F., Glazer, A. N. and Ames, B. N. (1987) Science **235**, 1043–1046

3 Keyse, S. M. and Tyrell, R. M. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 99–103

4 Schwertner, H. A., Jackson, W. G. and Tolan, G. (1994) Clin. Chem. **40**, 18–23

5 Wu, T.-W., Fung, K.-P. and Yang, C.-C. (1994) Life Sciences **54**, 477–481

6 Neuzil, J. and Stocker, R. (1994) J. Biol. Chem. **269**, 16712–16719

7 Rigney, E., Phillips, O. and Mantle, T. J. (1988) Biochem. J. **255**, 431–435

8 Rigney, E. and Mantle, T. J. (1988) Biochim. Biophys. Acta **957**, 237–242

9 Rigney, E., Mantle, T. J. and Dickinson, F. M. (1989) Biochem. J. **259**, 709–713

10 Colleran, E. and O'Carra, P. (1977) in Chemistry and Physiology of Bile Pigments (Berk, P. D. and Berlin, N. I., eds.), pp. 69–80, DHEW Publication No. (NIH) 77-1100

11 Phillips, O., Mantle, T. J., Tuffery, A. R., Heyworth, C. M., Wilson, S. R. and Houslay, M. D. (1984) Biochem. Pharmacol. **33**, 1963–1967

12 Yamaguchi, T., Komoda, Y. and Nakajima, H. (1994) J. Biol. Chem. **269**, 24343–24348

13 Yamaguchi, T., Komura, A., Nakano, Y., Tomita, M. and Nakajima, H. (1993) Biochem. Biophys. Res. Commun. **197**, 1518–1523

14 Maines, M. D. and Trakshel, G. M. (1993) Arch. Biochem. Biophys. **300**, 320–326

15 Sass, M. D., Caruso, C. J. and Farhangi, M. (1967) J. Lab. Clin. Med. **70**, 760–767

16 Xu, F., Quandt, K. S. and Hultquist, D. E. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 2130–2134

17 Reference deleted

18 O'Carra, P. and Colleran, E. (1969) FEBS Lett. **5**, 295–298

19 Ennis, O. and Mantle, T. J. (1995) Biochem. Soc. Trans. **23**, 443S

20 De Filippi, L. J. and Hultquist, D. E. (1978) J. Biol. Chem. **253**, 2946–2953

21 Laemmli, U. K. (1970) Nature (London) **227**, 680–685

22 Yubisui, T., Matsuki, T., Takeshita, M and Yoneyama, Y. (1979) J. Biochem. (Tokyo) **85**, 719–728

22a Chikuba, K., Yubisui, T., Shirabe, K. and Takeshita, M. (1994) Biochem. Biophys. Res. Commun. **198**, 1170–1176

23 Quandt, K. S. and Hultquist, D.E (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 9322–9326

Received 11 March 1996/29 March 1996; accepted 9 April 1996