HAEM OXYGENASE: A REAPPRAISAL OF THE STOICHEIOMETRY

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1. Introduction

Haem oxygenase (EC 1.14.99.3) in rat liver microsomes was first described as a mixed function oxidase utilising cytochrome *P*-450 [1]. Immunochemical studies suggest that NADP(H)-cytochrome *c* reductase activity is an essential component of the enzyme [2]. Indeed, it has been suggested [3] following the use of partially purified enzyme preparations, that all microsomal haem oxygenase activity could potentially be accounted for by the NADP(H)-cytochrome *c* reductase activity. This enzyme reduces added haem which then reacts directly with oxygen. A microsomal protein factor is required solely to provide specific cleavage at the α-methene bridge. Studies of a reconstituted haem oxygenase system [4] consisting of a partly purified haem oxygenase protein and a purified NADP(H)-cytochrome *c* reductase, indicated that the initial steps in the haem oxygenase reaction involved the binding of protohaem to haem oxygenase, followed by the reduction of the ferric haem by NADP(H)-cytochrome *c* reductase. The ferrous haem then reacts readily with molecular oxygen to cleave the α-methene bridge.

The mechanism of ring cleavage by haem oxygenase is still unclear however, although hydrogen peroxide, acting either directly, or through the action of hydroxyl-radicals, has been implicated [3]. Other workers have suggested the involvement of the superoxide anion [5,6] in this step. However, several workers have observed a lack of stoicheiometry in the haem oxygenase reaction [1,7], with indications that β-biliverdin may also be formed [8].

Abbreviations: MHA, methaemalbumin (Fe^{3+}); HA, haemalbumin (Fe^{2+}); E_h, redox potential

This study was undertaken to investigate the lack of stoicheiometry in the oxygenase assay and the role of NADP(H)-cytochrome *c* reductase in the reaction. An 18 000 × *g* liver supernatant was chosen as the enzyme source to ensure that if any parallel haem degradation mechanism was present [7] it would be detected by a deviation from the expected haem oxygenase stoicheiometry.

2. Materials and methods

Bovine haemin, α-bilirubin, α-biliverdin were obtained from Sigma Chemicals, Surrey. Catalase, glucose-6-phosphate dehydrogenase, $NADP^+$ and cytochrome *c* were purchased from Boehringer, Mannheim. All other chemicals were obtained from British Drug House, Dorset and were of the purest grade available.

2.1. *Preparation of 18 000* × g *liver homogenate supernatants*

Male rats (250–300 g, Wistar strain) were killed by cervical dislocation and the livers perfused in situ with 0.9% NaCl until bleached of haemoglobin. The perfused livers were homogenised in 2 vol. 0.25 M sucrose, centrifuged at 9000 × *g* (20 min) and the decanted supernatant fraction further centrifuged at 18 000 × *g* (10 min) and stored on ice until use.

2.2. *Assay for NADP(H)-cytochrome* c *reductase*

The activity of NADP(H)-cytochrome *c* reductase in 18 000 × *g* supernatants of rat liver homogenates was assayed by the method in [9].

2.3. *Preparation of methaemalbumin*

Methaemalbumin (MHA) solution was prepared as

in [1] but from bovine, instead of human serum albumin, to 1.8 mM final conc.

2.4. *Assay for haem oxygenase-biliverdin reductase*

The assay used was that in [2] but omitting the preincubation period, using a sensitive split-beam spectrophotometer capable of recording at 0.025 A units full scale [10]. A typical assay spectrum and rate of bilirubin formation (inset) is shown in fig.1.

2.5. *Measurements of rates of oxygen consumption or evolution*

Changes in the rate of oxygen uptake or evolution were measured in a Clark-type oxygen electrode, calibrated as in [11].

2.6. *Protein determination*

Protein concentrations were determined by the method in [12].

2.7. *Induction of haem oxygenase in rats*

Haem oxygenase was induced in rats by treatment with $CoCl_2$. Rats were given 3 subcutaneous injections (0.5 ml) of $CoCl_2 6H_2O$ (250 pmol/g body wt) in 0.9% NaCl at 12 h intervals; 12 h after the last injection the rats were killed and an 18 000 × g liver homogenate supernatant prepared as above.

2.8. *Stoicheiometry measurements*

Haem oxygenase was assayed as above; at the end of the experiments (immediately after the last spectrum) the cuvette contents were rapidly transferred to alkaline pyridine (final conc. pyridine 40% (v/v), NaOH 60 mM) and the MHA concentration in both reference and sample cuvettes determined as in [13].

3. Results

In the course of this study it became apparent that there were serious discrepancies between the amounts of haem disappearing and bilirubin being formed, under conditions of haem cleavage by haem oxygenase.

Figure 1 shows a typical set of spectra obtained in our standard assay. Bilirubin is identified by its broad absorption peak with A_{468} max and reduced cytochrome b_5 by the absorption peaks with A_{425} and

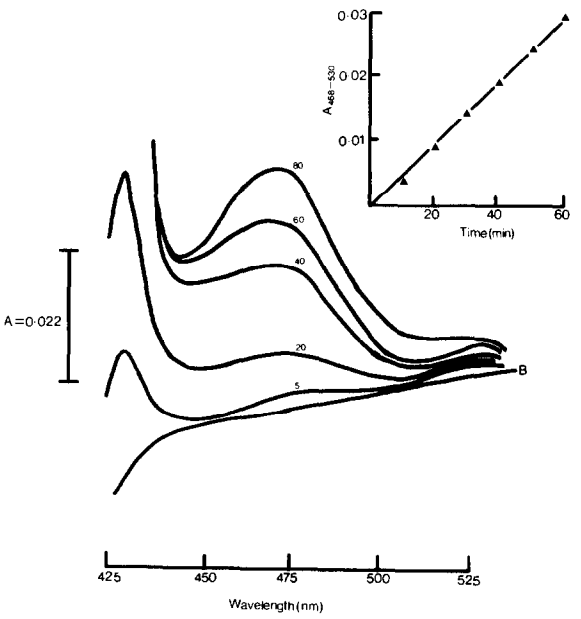

Fig.1. Spectral changes during a standard haem oxygenase–biliverdin reductase assay. To 2 ml of 18 000 × g perfused rat liver supernatant (final conc. 2 mg protein/ml) was added 0.32 ml 1.15% (w/v) KCl, 2 ml PO_4 buffer (pH 7.4) (0.2 M), 1 ml sucrose (0.25 M), bovine MHA 0.08 ml (prepared as in [1]), 0.2 ml glucose-6-phosphate (50 mg/ml), 0.2 ml glucose-6-phosphate dehydrogenase (140 IU/ml). After mixing rapidly, the preparation was divided between two cuvettes without further incubation, 0.1 ml 1.15% (w/v) KCl was added to the reference cuvette, 0.1 ml NADP (11.2 mg/ml in 1.15% KCl) was added to the sample cuvette and spectra recorded at the times shown (min). The A_{427} reflects the rapid reduction of cytochrome b. The insert shows the rate of bilirubin formation ($A_{465-530}$).

A_{525} max. The characteristics of the assay were qualitatively identical for 18 000 × g liver homogenate supernatants from control and $CoCl_2$-treated rats. The rate of production of bilirubin was linear for 50–60 min, after which time the reaction stopped, activity could be restored by vigorous stirring of the cuvette contents for 15–30 s. On average only 40% of the haem disappearing was recovered as bilirubin under standard assay conditions. We therefore re-examined the haem oxygenase reaction assay. The absence of biliverdin (A_{664} max) accumulation during the standard assay was shown by observation of the spectrum between 550 and 700 nm. To confirm that bilirubin was stable during the time course of the assays, we incubated

authentic bilirubin in our standard haem oxygenase assay. Over a 30 min. incubation period there was no change in the concentration of bilirubin (0.5–1.5 nmol/ml) in either the presence or absence of NADPH. Significantly, there was no change in the spectral characteristics of the bilirubin, which indicates that any conjugation reaction which might occur did not affect its spectral properties. Examination of the absorption spectra obtained during the standard assays, however, showed points where serious errors might arise:

1. Reduction of cytochrome b_5: this results in an increased absorbance close to 530 nm, commonly used as a reference wavelength in the assay. Over the course of the assay cytochrome b_5 became increasingly reduced, probably as a result of progressive lowering of the E_h in the cuvette.
2. A significant amount of MHA reduction may occur in the sample cuvette (+NADPH) during the assay, causing a broad trough centred at 490 nm to be formed. This tends to diminish the absorbance at 468 nm due to bilirubin formation.

On prolonged incubation of the assay mixture an apparent loss of bilirubin was observed (fig.2). This is apparently due to the factors outlined above since vigorous stirring rapidly restored the bilirubin spectrum. Compensating for the errors arising from (1) and (2) above, it was possible to achieve a consistent improvement in yield of bilirubin to ~70% of the haem used by extracts of control animals (based on 20 determinations). Addition of EDTA (1 mM) to the reaction mixture, to prevent haem destruction by lipid peroxidation, did not increase the product yield. However, when $CoCl_2$-treated rats were used, a 100% yield was achieved (3 determinations).

Two major causes of low yield were considered possible in extracts from control animals:

(i) The breakdown of haem by NADPH-cytochrome *c* reductase [3];
(ii) The direct action of endogenously-produced hydrogen peroxide in breaking down haem.

Under the conditions used in our standard haem oxygenase assay the specific activity of NADP(H)-cytochrome *c* reductase was 31 nmol cytochrome *c* reduced .min^{-1} .mg $protein^{-1}$ and of haem oxygenase was 5.4 nmol .h^{-1} .mg $protein^{-1}$. We could monitor the activity of the two enzymes of the assay mixture individually, under standard assay conditions, by

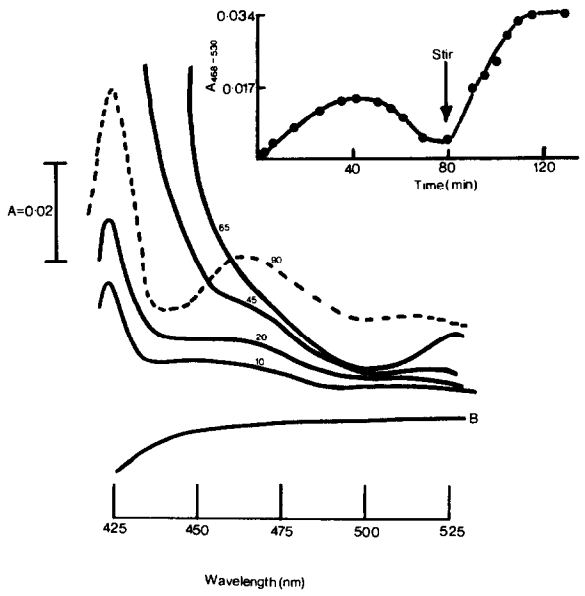

Fig.2. Apparent loss of bilirubin during a standard haem oxygenase–biliverdin reductase assay. A standard assay mixture was prepared as in fig.1 (but at 3 mg protein/ml). Spectra were recorded at the times shown (min). The inset shows the $A_{486-530}$ against time. The dotted line shows the effect of vigorous stirring at t = 80 min.

bubbling with carbon monoxide for 1 min prior to assay. Haem oxygenase activity was then completely inhibited but the activity of NADPH-cytochrome *c* reductase was unaltered from 31 nmol cytochrome *c* reduced .min^{-1} .mg $protein^{-1}$. With this CO-treated system the rapid appearance of a peak at 450 nm was observed, due to the formation of a reduced cytochrome *P*-450–CO complex, followed by a slower absorbance increase at 425 nm, produced by the reduction of cytochrome b_5 on addition of NADP(H). No bilirubin was produced, since no A_{468} (due to bilirubin) was noted, even after 1 h incubation.

Using a standard assay mixture, but without added MHA, treatment with carbon monoxide and reduction with dithionite resulted in a spectrum identical in form to that of the CO-inhibited assay mixture. However the $\Delta A_{425-412}$ was 0.064, compared with 0.12 in the assay mixture containing MHA. Thus the reduction of cytochrome b_5 cannot be solely responsible for the increased A_{425} found in the CO-inhibited assay, there must be a contribution from MHA. A

solution of MHA, of the same concentration as that used in the standard assay, reduced with dithionite and bubbled with CO produces an A_{425} peak with an $A_{425-412}$ 0.22. This suggests that in the assay mixtures, after the initial reduction of cytochrome b_5, some reduction of MHA occurs. Not all the MHA in solution is reduced, since a total $\Delta A_{425-412}$ ~0.3 would then be expected. The midpoint oxidation–reduction of MHA in free solution, was determined as −50 mV at pH 7.0 (G. A. F. H., O. T. G. J., unpublished results) compared with +15 mV for cytochrome b_5 [14] which is consistent with this pattern of reduction.

To determine whether in the CO-inhibited system the main product of reaction was a biliverdin, but not the α-isomeric form, the above experiment was repeated and the spectra of the cuvette contents scanned between 550–700 nm (biliverdin, A_{664} max). During the course of the assay a compound was produced with an A_{658} max which was quite distinct from the absorbance maximum produced on addition of authentic biliverdin. This spectrum of added biliverdin showed no change over a further 1.5 h. Unlike authentic biliverdin the species with A_{658} max was unstable in alkaline pyridine and in pyridine. An A_{658} peak was also found to occur in our standard assay mixture omitting the CO treatment.

The reduction of MHA by the CO-treated standard assay mixture was interesting, since it has been proposed that the product, HA, is the form in which haem reacts with the enzyme haem oxygenase [8] and that in the presence of hydrogen peroxide it degrades spontaneously to biliverdin [3]. However, in assessing the contribution of NADPH-cytochrome *c* reductase to haem degradation in our system, the lack of biliverdin or bilirubin production even when the MHA is seen to be reduced could have been due to an absence of oxygen in the cuvette following CO flushing. An alternative method of producing reduced MHA under assay conditions was thus sought. We were able to produce HA under conditions of the standard assay, if the liver 18 000 × *g* supernatants were stored before assay.

Supernatants of rat liver homogenates, stored at 0°C for 24 h or longer show almost complete loss of haem oxygenase activity, while retaining full NADP(H)-cytochrome *c* reductase activity. After 10–15 min incubation, under standard assay conditions, there was an increased $A_{425-530}$ and the production of a trough at 490 nm. This change is greater than that produced by complete reduction of the cytochrome b_5 of the extract and is associated with the reduction of MHA. Vigorous stirring of the cuvette contents resulted in a re-oxidation of MHA, continued reduction of cytochrome b_5 but no significant increase in A_{468} which would be given by bilirubin.

A central role in proposed haem oxygenase reaction schemes has been ascribed to hydrogen peroxide [3]. Our standard 18 000 × *g* supernatants from rat liver homogenates were found to contain significant amounts of catalase-type activity. On addition of 22 μmol hydrogen peroxide to 1 ml of a suitably diluted supernatant sample, oxygen was evolved at 0.12 mg atoms .mg protein^{-1} .min^{-1}. A rate of 0.5 mg atoms .mg protein^{-1} .min^{-1} was obtained for pure catalase, and the high catalatic activity of the 18 000 × *g* supernatants would seem to exclude the possibility that hydrogen peroxide, in free solution, is involved in the enzyme-catalysed reactions or haem degradation per se under standard assay conditions.

The absence of a 100% yield of biliverdin may indicate the presence of a second, but minor, haem degradative mechanism in normal rats. This secondary pathway may account for 30% of haem breakdown in control rats. In $CoCl_2$-induced animals however, this second mechanism may be suppressed or be simply undetectable in competition with a Co-induced increase in haem oxygenase activity. Indications that haem (as MHA) is degraded to compounds other than biliverdin/bilirubin have been described in photosynthetic bacteria and higher plant microsomes [15]. Similarly, cytochrome *c* appears to be degraded in the dog and rat to unknown compounds [7].

4. Discussion

The findings reported here confirm earlier observations [1,4,8] that there is a discrepancy between the amount of haem disappearing and bilirubin appearing during haem breakdown catalysed by haem oxygenase. In view of the possible formation of non α-isomers of biliverdin or bilirubin, as suggested [8], we investigated the stoicheiometry of haem oxygenase more closely.

The assay for this enzyme is a linked assay in which the product of haem oxygenase, biliverdin,

which has a small A_{664} peak (E_{mM} = 8 $mM^{-1}l^{-1}$) [2] is converted to a more highly absorbing species, bilirubin, with an A_{468} peak (E_{mM} = 57 $mM^{-1}l^{-1}$) [2]. The reaction which converts α-biliverdin to bilirubin is not rate limiting and proceeds with the expected stoicheiometry [1]. Liver 18 000 × *g* supernatants containing microsomal electron-transport components are used as a source of both haem oxygenase and α-biliverdin reductase, and we have found that serious errors are introduced into the assay by the spectrum of reduced cytochrome b_5 (which has an A_{530} peak, used as a reference wavelength). The rapid reduction of this cytochrome can give rise to an artefactual lag-phase (see [1]) at the start of the reaction, due to an increase in absorbance of the β-peak of cytochrome b_5 at 525 nm, within 15 s of adding NADP(H). A more serious source of error, however, is introduced into the assay by the substrate bovine MHA. Under certain conditions reduction of MHA occurs, resulting in a 2–3-fold increase in $A_{425-430}$ in difference spectra and production of a broad trough with a minimum at 490 nm; this latter effect results in an apparent loss of bilirubin from the reaction mixture. When the spectral artefacts were eliminated a 70% yield (instead of 40%) was obtained from the haem cleavage reaction. From spectra taken between 550 and 700 nm we found no evidence for the formation of bilirubin isomers, other than the α-isomer. An unexpected peak, not associated with either the 18 000 × *g* supernatant from rat liver homogenates or MHA was observed at 658 nm during reaction. The absorbing species was unstable in both pyridine and alkaline pyridine, in contrast to the authentic α-IX biliverdin (A_{664} max) which was stable in both these solvents. This new absorbing species was, however, stable to vigorous aeration. We have yet to identify the cause of this absorption. Measurements of the stoicheiometry of the oxygenase reaction in extracts from $CoCl_2$-treated rats, to induce haem oxygenase [16], gave ratios of substrate to product of 1:1. In view of these facts, we conclude that there is little evidence for the formation of significant amounts of isomers other than α-IX biliverdin under $CoCl_2$ treatment conditions, but under normal conditions the lack of stoicheiometry would suggest an alternative pathway of haem breakdown is present in the liver.

The importance of NADPH-cytochrome *c* reductase in haem degradation has been reported [2]. We have found that haem oxygenase is completely inhibited by CO, while leaving NADPH-cytochrome *c* reductase fully active. Such a system shows no detectable haem degrading capacity, as determined by either ΔA_{468} or ΔA_{664}. The CO-inhibited system did however retain the ability to reduce MHA. Confirmation that NADPH-cytochrome *c* reductase activity alone was insufficient for haem oxygenase activity was obtained using freeze-stored 18 000 × *g* supernatants of liver homogenates in which all haem oxygenase activity was lost but which retained full cytochrome *c* reducing capacity. These were not able to break down haem.

The measurement of catalase-type activity in the rat liver 18 000 × *g* supernatants routinely used showed that hydrogen peroxide in free solution is unlikely to be present under our standard assay conditions and thus could not be responsible for minor haem losses.

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