Cloning, sequencing and expression of cDNA for chick liver haem oxygenase

Comparison of avian and mammalian cDNAs and deduced proteins

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A cDNA from a chick liver library that encodes for haem oxygenase has been cloned and sequenced. Positive clones were identified with monospecific antibodies to the purified enzyme from chick liver and a cDNA of rat haem oxygenase-1. The length of the cDNA is 1258 bases. An open reading frame of 888 bases was identified by comparison of nucleotide and amino acid sequences with those previously identified for haem oxygenase of mammalian or avian origin. The protein corresponding to this fragment of DNA is composed of 296 amino acid residues and has a molecular mass of 33 509 Da, which is similar to that previously estimated for haem oxygenase purified from chick liver. Unequivocal identification of this clone as that complementary to haem oxygenase was provided by (a) comparison of amino acid compositions and partial sequences with those previously established for the purified enzyme, (b) comparison with nucleotide and amino acid sequences for haem oxygenase from rat and human sources and (c) expression in *Escherichia coli* with production of high levels of mRNA, protein and haem oxygenase activity after exposure of the transfected bacteria to isopropyl β -D-thiogalactopyranoside (IPTG). Overall, the similarity of chick haem oxygenase to rat and human haem oxygenase (nucleotides 66 % and amino acids 62 %) is moderately high. The region between proline-129 and alanine-157 is identical in all three enzymes, including histidine-135, which is proposed to play a key role in binding the substrate haem at the active centre of the enzyme. Northern blots also show that treatment of chicks with CdCl₂, a potent inducer of haem oxygenase, results in increases in 1.65–1.70 kb mRNA, which hybridizes selectively to the full-length cDNA or to a synthetic 24-base oligonucleotide with sequence identical to that of a portion of the haem oxygenase cDNA. These results suggest that Cd-dependent induction of haem oxygenase is due to increased transcription of the gene or stabilization of its message.

INTRODUCTION

Haem oxygenase (EC 1.14.99.3) catalyses the specific cleavage of haem at the α -methene bridge, with formation of stoichiometric amounts of biliverdin IX α , CO and iron [for reviews, see Kikuchi & Yoshida (1983), Abraham *et al.* (1988) and Maines (1988)]. For some time it has been generally accepted that the haem of senescent erythrocytes is broken down by haem oxygenase (Bissell *et al.*, 1977); however, the fate of haem synthesized by hepatocytes, chiefly for incorporation into cytochrome(s) P-450 (Marver & Schmid, 1972) has been less certain (Bissell & Guzelian, 1980; Grandchamp *et al.*, 1981). The uncertainty regarding the fate of haem synthesized by hepatocytes prompted us to investigate the question using primary cultures of chick-embryo liver cells. These cultures are useful because they maintain normal levels and inducibility of cytochrome(s) P-450 (Sinclair *et al.*, 1979, 1989; Bonkovsky *et al.*, 1987), haem oxygenase (Maines & Sinclair, 1977; Sardana *et al.*, 1982; Lincoln *et al.*, 1988) and 5-aminolaevulinic synthase, the rate-controlling enzyme for haem synthesis (Granick *et al.*, 1975; Srivastava *et al.*, 1980).

In contrast with results reported from studies with primary cultures of rat hepatocytes (Bissell & Guzelian, 1980), in chick-embryo or adult-rat liver cell cultures, we found that all haem catabolism occurred by action of haem oxygenase, with formation of stoichiometric amounts of biliverdin and CO (Lincoln *et al.*, 1989). We also showed that there are at least two distinct mechanisms for induction of haem oxygenase in these cultures,

one dependent on an increased supply of haem (produced by haem or phenobarbitone-like drug administration) and the other independent of haem synthesis (produced by Co or Cd administration) (Lincoln *et al.*, 1988; Cable *et al.*, 1990), in agreement with conclusions reached from studies in intact rats (Kikuchi & Yoshida, 1983).

Following the demonstration that haem oxygenase plays the critical role in regulation of physiological haem catabolism by hepatocytes, we purified and characterized the oxygenase from chick livers (Bonkovsky *et al.*, 1990) and prepared antibodies to the enzyme (Y. Greene, J. Healey, C.-O. Evans & H. L. Bonkovsky, unpublished work). To gain further insight into the factors that regulate expression of hepatic haem oxygenase and the molecular mechanisms underlying changes in its expression, we have used the antibodies to clone cDNA from the chick that codes for the major inducible form of haem oxygenase present in liver (Shibahara *et al.*, 1985; Maines, 1988). We have determined the nucleotide sequence of this clone. In this paper we describe these studies and compare the structures of the cDNA and deduced peptides of chick and mammalian haem oxygenases (Shibahara *et al.*, 1985; Maines, 1988; Shibahara, 1988; Yoshida *et al.*, 1988).

MATERIALS AND METHODS

Materials

A λ ZAP chicken liver cDNA library and pBluescript SK(–)

Abbreviations used: IPTG, isopropyl β -D-thiogalactopyranoside; LB, Luria–Bertani broth (in 1 litre: 10 g of Bacto-tryptone, 5 g of yeast extract, 10 g of NaCl, pH adjusted as needed to 7.5 with NaOH); PBKCl, 0.01 M-potassium phosphate/0.15 M-KCl, pH 7.4; 1 $\times$ SSC, 0.15 M-NaCl/0.017 M-sodium citrate, pH 7.0; poly(A)⁺, polyadenylated.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases.

were purchased from Stratagene (La Jolla, CA, U.S.A.). cDNA of rat haem oxygenase was kindly donated by Professor S. Shibahara, Tohoku University, Sendai, Japan. Restriction endonucleases were obtained from Promega (Madison, WI, U.S.A.). The goat anti-rabbit IgG conjugated with alkaline phosphatase, the alkaline phosphatase development kit, DNA-grade agarose, ammonium persulphate, nitrocellulose blotting paper (0.45 μ m pore size) for Western-blot analysis were from Bio-Rad (Richmond, CA, U.S.A.). Nitrocellulose filters (0.45 μ m pore size) for the screening of the library were from Vanguard International (Neptune, NJ, U.S.A.). The hybridization nylon membranes (Gene Screen) were from NEN Research Products (Wilmington, DE, U.S.A.). RNazol was purchased from CINNA/BIOTECX Laboratories International (Friendswood, TX, U.S.A.). [γ - 32 P]ATP (3000 Ci/mmol), [α - 32 P]dCTP (3000 Ci/mmol) and [α - 35 S]thio-dATP (1000 Ci/mmol) were from Amersham Corp. (Arlington Heights, IL, U.S.A.). T7 DNA polymerase (Sequenase) was from United States Biochemical (Cleveland, OH, U.S.A.). T3, T7 and other oligonucleotide primers and probes were made by the Microchemical Facility at Emory University (Atlanta, GA, U.S.A.). Horizontal-gel-electrophoresis apparatus model H5, DNA-sequencing gel-electrophoresis apparatus model S2 and RNA size markers ('RNA ladder') were from Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.). Isopropyl β -D-thiogalactopyranoside (IPTG) and protein molecular-mass markers were from Sigma (St. Louis, MO, U.S.A.). Poly(ethylene glycol) 8000 (PEG; M_r 7000–9000) was from Fisher Scientific (Atlanta, GA, U.S.A.). NN'-Methylenebisacrylamide was from Pierce Chemical Co. (Rockford, IL, U.S.A.). Urea was from Mallinckrodt (St. Louis, MO, U.S.A.). The PhastSystem and gradient gels were from Pharmacia-LKB Biotechnology (Piscataway, NJ, U.S.A.). Other chemicals were of the highest purity commercially available.

Buffers

The 3 $\times$ sample buffer for Western blotting was prepared by mixing 5 vol. of 40 mM-Tris/HCl/4 mM-EDTA, pH 8.0, with 1 vol. of 10% (w/v) SDS in β -mercaptoethanol and 1 vol. of

0.1% Bromphenol Blue in aq. 15% (v/v) glycerol. The Hepes buffer used for Northern blotting was 20 mM-Hepes/5 mM-sodium acetate/1 mM-EDTA, pH 7.2.

Methods

Preparation of antibodies to chick haem oxygenase. Haem oxygenase was purified (Bonkovsky *et al.*, 1990) and injected into female New Zealand White rabbits (Kaminsky *et al.*, 1981). After suitable intervals, booster injections of antigen were given, rabbits were bled, and sera recovered by centrifugation. IgG was prepared from the sera (McKinney & Parkinson, 1987), from which monospecific antibodies were prepared (Girard *et al.*, 1986).

Screening of cDNA library and cloning of cDNA for chick haem oxygenase. A chick liver cDNA library, in λ ZAP vector, was screened using affinity purified IgG to chicken haem oxygenase. *Escherichia coli* (BB4 strain) was infected with the library and plated to a density of 500 000 plaque-forming units/150 mm-diameter Luria-Bertani broth (LB) plate. The plates were incubated at 42 °C for 6 h or until plaques appeared, and were then overlaid with nitrocellulose filters saturated with 10 mM-IPTG and incubated for an additional 3.5 h at 37 °C. After being blocked with 5% (w/v) non-fat milk and 0.1% NaN₃ in PBKCl, nitrocellulose filters were exposed to anti-(chicken haem oxygenase) affinity-purified antibody, and the positive plaques were revealed by means of a second antibody, namely goat anti-rabbit IgG conjugated with alkaline phosphatase (for details, see under 'Western-blotting analysis' below). Potential positive clones were screened an additional four times to achieve 100% purity. The cloned cDNA insert in the λ ZAP vector was automatically excised, and re-circularized into a smaller phagemid [pBluescript SK(–)] using an f1 helper phage (Short *et al.*, 1988). After the recircularized phagemid had been secreted, the *E. coli* cells were heated at 70 °C for 20 min and centrifuged (4000 g, 5 min). The supernatant from this spin, which contains the pBluescript plasmid, was incubated with fresh *E. coli* cells (XL1-Blue) according to the manufacturer's protocol (Stratagene Cloning

Fig. 1. Sequencing strategy and restriction map of cloned cDNA encoding chick haem oxygenase

The protein-coding region is indicated by an open box, and the 5' and 3' noncoding regions are shown by the cross-hatched boxes. The arrows indicate the directions and extent of sequence determinations. The arrows directed inward from the 5' (left) and 3' (right) termini indicate reactions using T₃ and T₇ sequencing primers respectively. All other sequencing reactions were primed with synthetic oligonucleotides complementary to previously determined cDNA sequences. The restriction map shows only selected relevant sites.

Systems, 1988). For production of double-stranded DNA, the transformed *E. coli* were grown on plates containing LB/ampicillin. Transformation (Ausubel *et al.*, 1989) of XL1-Blue with pBluescript SK(–) without insert was also performed for use as a control. Mini-Preps and Maxi-Preps [Maniatis *et al.* (1982), pp. 90–91, 93–94 and 366–367] of pBluescript with or without insert were performed, and the resulting cDNA insert was released by digestion with endonuclease *EcoRI*. The *EcoRI*-digested pBluescript DNA with or without insert was subjected to electrophoresis on a 1% (w/v)-agarose gel containing ethidium bromide [Maniatis *et al.* (1982), p. 161], and the existence of an

EcoRI fragment insert was confirmed. The identity of the cloned insert was confirmed by hybridization of a Southern blot with ³²P-labelled *EcoRI*–*Hind* III, fragment of rat haem oxygenase cDNA (Shibahara *et al.*, 1985). This hybridization was carried out overnight at 65 °C, followed by one wash with 1 × SSC (room temperature; 20 min) and then four washes with 0.1 × SSC (55 °C, 30 min). The cDNA of chicken liver haem oxygenase was isolated by low-melting-temperature-agarose-gel electrophoresis followed by elution from the gel with butan-1-ol extraction and ethanol precipitation [Maniatis *et al.* (1982), p. 377].

DNA sequence analysis. Nucleotide sequences of the cDNA inserts were determined by using a modification of the dideoxyribonucleotide method (Sanger *et al.*, 1977), which uses strand-specific primers (T3, T7 and other specially synthesized oligonucleotide primers), [α -³⁵S]dATP and modified T7 DNA polymerase (Sequenase). The sequencing was performed in both directions directly from the double-stranded plasmid DNA (Zhang *et al.*, 1988) obtained from Maxi Preps. Comparisons of nucleotide and deduced amino acid sequences with those for other enzymes were performed using GENEPRO software (Riverside Scientific Enterprises, Seattle, WA, U.S.A.).

Expression of haem oxygenase. *E. coli* (XL1-Blue) were transfected with pBluescript SK(–), either with or without the cDNA insert for chick liver haem oxygenase. Selected isolated colonies were inoculated and grown at 30 °C in 600 ml of super broth (containing, in 1 litre: 5 g of NaCl, 20 g of Bacto-yeast extract, 35 g of tryptone, 1 ml of 5 M-NaOH and 100 mg of ampicillin) to an absorbance of 0.5 at 600 nm. Thereafter, the temperature was increased as quickly as possible to 42–45 °C for 20 min. IPTG was then added to a concentration of 10 mM, and the cells were incubated at 37 °C for a further 6 h. Aliquots of cells were taken at 0, 1, 3 and 6 h after IPTG was added, for determination of haem oxygenase activity, Western-blot analysis and isolation of total RNA for Northern-blot analysis.

Table 1. Comparison of amino acid composition of chick liver haem oxygenase from deduced sequence and purified enzyme

Amino acid	Composition (mol/mol of protein)	
	Deduced (the present work)	From purification (Bonkovsky <i>et al.</i> , 1990)
Ala	30	24
Arg	13	16
Asn	12	18
Asp	7	
Cys	1	ND*
Gln	15	34
Glu	29	
Gly	13	21
His	10	9
Ile	6	10
Lys	25	26
Leu	33	31
Met	9	5
Phe	14	14
Pro	12	13
Ser	20	23
Thr	16	15
Trp	2	ND
Tyr	10	9
Val	19	20

* ND, not determined.

-68 CCCAGGCTTC

ATTGGATTTC AAGGAACAA CAGGGAAGCT CGAGAGGCTC GAGACCGGA GGAGAAGC

ATG GAA ACT TCG CAG CCA CAC AAC GCT GAA AGC ATG TCC CAG GAC 45
M E T S Q P H N A E S M S Q D 15

CTG TCG GAG CTC CTG AAG GAA GCC ACC AAG GAG GTG CAC GAG CAG 90
L S E L L K E A T K E V H E Q 30

GCG GAG AAC ACA CCC TTC ATG AAG AAC TTC CAG AAG GGA CAG GTG 135
A E N T P F M K N F E K G Q V 45

TCC CTC CAC GAG TTC AAG CTG GTC ACG GCG TCC CTG TAC TTC ATC 180
S L H E F K L V T A S L Y F I 60

TAC TCT GCT CTG GAG GAA GAG ATC GAA CGT AAC AAG GAC AAC CCT 225
Y S A L E E E I E R N K D N P 75

GTC TAT GCT CCT GTT TAT TTT CCG ATG GAG CTG CAC CGG AAA GCT 270
V Y A P V V F M E L H R K A 90

GCC CTG GAG AAA GAC TTG GAG TAC TTC TAT GGC AGC AAC TGG AGG 315
A L E K D L E Y F Y G S N W R 105

GCA GAG ATC CCA TGT CCT GAG GCT ACT CAG AAA TAC GTT GAG AGG 360
A E I P C P E A T Q K Y V E R 120

CTC CAC GTC GTT GGC AAG AAG CAT CCA GAG CTG TTG GTG GCC CAT 405
L H V V G K K H P E L L V A H 135

GCC TAC ACC CGC TAT TTG GGA GAC CTG TCT GGG GGG CAG GTG CTG 450
A Y T R Y L G D L S G G Q V L 150

AAG AAA ATC GCC CAA AAG GCC CTG CAG CTG CCC AGC ACT GGA GAA 495
K K I A Q K A L C Q L P S T G E 165

GGG TTG GCT TTC TTC ACC TTC GAT GGA GTC TCC AAC GCC ACC AAG 540
G L A F F T F D G V S N A T K 180

TTC AAA CAG CTC TAT CGG TCC CGA ATG AAT GCC CTT GAG ATG GAT 585
F K Q L Y R S R M N A L E M D 195

CAT GCT ACT AAG AAA AGA GTC TTG GAG GAA GCC AAG AAA GCA TTC 630
H A T K K R V L E E A K K A F 210

CTG CTA AAT ATA CAG GTG TTT GAG GCG CTG CAG AAG CTG GTG TCC 675
L L N I Q V F E A L Q K L V S 225

AAG AGC CAG GAG AAC GGT CAT GCT GTA CAG CCA AAG GCA GAG CTT 720
K S Q E N G H A V Q P K A E L 240

CGC ACA AGG AGT GTT AAC AAA TCG CAT GAA AAC AGT CCA GCA GCT 765
R T R S V N K S H E N S P A A 255

GGG AAG GAG AGT GAG AGG ACA AGC AGG ATG CAG GCA GAC ATG CTG 810
G K E S E R T S R M Q A D M L 270

ACC ACC TCT CCC CTG GTG CGG TGG CTC CTG GCG CTC GGC TTC ATC 855
T T S P L V R W L L A L G F I 285

GCC ACC ACA GTC GCC GTG GGC TTA TTT GCC ATG TGA G CGAGCAGGAC 902
A T T V A V G L F A M * 296

CTCCGCTTC TCCTGTGTC TTCCCTCACC CTGAACATA CTACTTTGCC CAGTCTTCC
AAGAAATCC CTTCGACTGG GCTCTGCCAC TAATATTTCG AGACCTTCT CCCAGAAAG
CAGAGTCTG CGAGGGGCTC CAGGCCCTTT CCTGCTTGC TGCAAGCTCT CTTCAACAC
TAACACATC ACCACAGGC CCAAGGATG GAAAGGCTC AAACCAACAG CCAAGAGCTG
CAGTTATTCT ACAGAGTATC TGCAAAATC CCAAGGTGT CAAAGGG 1190

Fig. 2. Nucleotide sequence of cDNA encoding chick haem oxygenase and its deduced amino acid sequence

The nucleotide sequence of the message strand is shown. Nucleotides are numbered in the 5'-to-3' direction. Nucleotide residue 1 is the A of the initiating methionine codon ATG. Nucleotides 5' to the initiation codon are indicated by negative numbers. The deduced amino acids are shown by the single-letter code below the nucleotide sequence and are numbered beginning with the initiating methionine residue. The putative membrane-bound segment is underlined. * indicates the TGA stop codon.

Measurements of haem oxygenase activity. For determination of haem oxygenase activity, 20–40 ml of cells were centrifuged at 4400 *g* for 5 min and the pellet resuspended in 2 ml of 0.1 M-potassium phosphate, pH 7.40. A 100 μ l aliquot was mixed with 50 μ l of electrophoresis sample buffer for Western-blot analysis (see below). The remainder was sonicated twice with 5 s bursts of a W-225 sonicator equipped with a microtip (Heat Systems Ultrasonics, Farmingdale, NY, U.S.A.) set at a power level of 4. The sonicated material was centrifuged at 10000 *g* for 5 min, and the supernatant used for determination of haem oxygenase activity, which was measured by using the dual-wavelength assay described previously (Bonkovsky *et al.*, 1990). The reaction mixture, in a final volume of 1 ml, contained 70 μ mol of potassium phosphate buffer, pH 7.40, 5 μ mol of deferoxamine, 0.6 unit of purified NADPH:cytochrome reductase, 50 μ l of rat liver supernatant (105000 *g*) as a source of biliverdin reductase, 1 mg of egg phosphatidylcholine, 42 nmol of haem added as a methaemalbumin solution and 25–500 μ g of *E. coli* supernatant protein. Protein was determined using the bicinchoninic acid method (Smith *et al.*, 1985).

Western-blotting analysis. For Western blotting, 100 μ l of

resuspended cells was mixed with 50 μ l of 3 $\times$ sample buffer, then boiled for 5 min to denature proteins completely. The final suspension was diluted to 1 mg of protein/ml in 1 $\times$ sample buffer, and 1 μ l was used for electrophoresis. Proteins were separated on 8–25 % gradient gels in the PhastSystem, according to the manufacturer's specifications. For transfer of proteins from the Minigels to nitrocellulose paper (0.45 μ m pore size), gels were placed into Petri dishes and covered with a sheet of nitrocellulose, three sheets of blotting paper and a metal block weighing 68 g. These transfers were carried at 70 °C for 2 h in the presence of a buffer with the following composition: 25 mM-Tris/HCl (pH 9.2)/192 mM-glycine/5 % (v/v) methanol. Before use, the nitrocellulose and blotting papers were pre-soaked for 10 min in the same buffer.

After transfer, the nitrocellulose was blocked overnight in buffer containing PBKCl/1 % (w/v) BSA. This and all subsequent incubations were completed with gentle shaking at room temperature. After blocking, the nitrocellulose was rinsed with distilled water and then incubated for 4 h in primary anti-(haem oxygenase) antibody [affinity-purified antibody was diluted to approx. 25 mg of IgG/ml in PBKCl containing 1 % (w/v)

Fig. 3. Comparison of the amino acid primary and secondary structures of chick (cHO), rat (rHO-1) and human (hHO-1) haem oxygenases

The amino acids are shown by the single-letter code and have been aligned to provide optimal similarity among the three proteins. The numbering scheme follows the order for the chick enzyme. The shaded areas indicate the regions of sequence identity among the three proteins. Note that the three are identical between residues 129 and 157, a region that includes His¹³⁵, previously suggested to play a key role in substrate binding (Bonkovsky *et al.*, 1990). Predicted secondary structures are shown as follows: —, turns; ---, α -helical regions; ~~~, β -sheet regions. Predictions were done with the algorithm of Chou & Fasman (1978) in GENEPRO software.

BSA/0.02% Na₂S₂O₅. The nitrocellulose was washed three times, 15 min each, with PBKCl/0.5% (w/v) Tween-20 and then rinsed with distilled water. A second antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG) was diluted 1:2000 in PBKCl and applied to the nitrocellulose for 90 min; thereafter the paper was washed as described above.

Western blots were developed using an alkaline phosphatase conjugate substrate kit as recommended by the manufacturer (Bio-Rad, Richmond, CA, U.S.A.).

DNA probe preparation and Northern-blot analysis. ³²P-labelled cDNA probes were prepared (Feinberg & Vogelstein, 1984) using restriction fragments isolated from low-melting-temperature agarose gels. After the cDNA sequence had been determined, an oligonucleotide of 24 bases (nucleotides 533–557) was synthesized, 5'-end-labelled with [γ -³²P]ATP (Hamilton *et al.*, 1988) and used as a probe for Northern analysis. Total RNA for Northern blotting was isolated from tissue and cell culture by the RNazol method (Chomczynski, 1988), a modification of the one-step acid guanidinium thiocyanate/phenol/chloroform procedure of Chomczynski & Sacchi (1987). Poly(A)⁺ RNA was isolated using oligo(dT)-cellulose column chromatography [Davis *et al.* (1986), pp. 139–142]. Total RNA and poly(A)⁺ RNA were denatured and electrophoresed in 1.2% (w/v) agarose gel containing 2.2 M-formaldehyde. After electrophoresis (approx. 2 h at 150 V in Hepes buffer), RNA was capillary-blotted in presence of 20×SSC for 18 h on to Gene Screen nylon membranes according to the manufacturer's recommendations. The membranes were washed with 0.025 M-sodium phosphate, pH 6.5, to remove all the agarose debris, baked for 1 h at 80 °C and then prehybridized as previously described (Li *et al.*, 1989). The hybridization with the oligonucleotide probe (5 × 10⁶ c.p.m. in a 20 ml bag) was carried out at 65 °C overnight. Then the membranes were washed once with 1×SSC, followed by three washes with 0.1×SSC (each wash was for 20 min at room temperature). Membranes were then exposed to Kodak X-ray film with an intensifier screen at –80 °C for 2–3 days before the films were developed.

RESULTS AND DISCUSSION

Sequencing of chick haem oxygenase

Three clones that produced proteins that reacted strongly with antibodies against chick haem oxygenase, and DNA that hybridized with cDNA of rat haem oxygenase, were selected for further study. The sizes of the cDNA inserts were estimated by electrophoresis, and the longest of the three (~1.3 kb) was amplified and sequenced. The full coding region of the cDNA was sequenced at least twice in both the 5'-to-3' and 3'-to-5' directions. Any areas of ambiguity were sequenced additional times, as needed, until confident assignment of primary structure could be made.

Fig. 1 shows the sequencing strategy and selected restriction-endonuclease sites of the cloned cDNA, and Fig. 2 shows the full nucleotide sequence of the cDNA as well as its deduced amino acid sequence. The putative ribosomal binding site (AGGAG...) (Watson *et al.*, 1987) is located 9 bases upstream of the initiation codon. The deduced protein contains 296 amino acid residues and has a molecular mass of 33 509 Da, in good agreement with the mass estimated from study of the purified protein (Bonkovsky *et al.*, 1990). As Table 1 shows, close agreement also exists between the previously reported amino acid composition of the purified protein (Bonkovsky *et al.*, 1990) and the composition from the deduced amino acid sequence. The slight differences generally are within experimental error for determination of amino acid compositions; the discrepancy in content of glutamic

acid and glutamine (Glx) probably relates to underestimates due to the well known instability of the phenylthiocarbamoyl derivative of glutamic acid (Applied Biosystems, 1988), whereas the discrepancy in glycine content may have been due to residual contamination of the hydrolysed protein by glycine from one of the buffers used in purification and preparation of the protein.

The chick haem oxygenase has a fairly high degree of similarity to forms of haem oxygenase previously cloned and sequenced

Fig. 4. Expression of chick haem oxygenase protein in *E. coli* transfected with pBluescript with or without chick haem oxygenase cDNA insert

E. coli (XL1-Blue) was transfected with pBluescript either with or without the cDNA insert for chick haem oxygenase, grown for 0–6 h in the presence of IPTG portions, harvested and assayed as described in the Materials and methods section. A 1 µg portion of sonicated protein was subjected to Phast-gel electrophoresis and Western blotting as described in the Materials and methods section. The Western blot was constructed as follows: lane 1, 15 ng of highly purified haem oxygenase; lanes 2–4, 50 ng of total protein from *E. coli* cells which had been transfected with pBluescript lacking the cDNA insert exposed to IPTG for 1, 3 and 6 h respectively; lanes 5–7, 50 ng of total protein from *E. coli* cells transfected with pBluescript containing the cDNA insert for haem oxygenase exposed to IPTG for 1, 3 and 6 h respectively.

Fig. 5. Autoradiogram of Northern Blot of total RNA of *E. coli* transfected with pBluescript with or without chick haem oxygenase cDNA insert

Lanes A–D contained 1 µg of total RNA of *E. coli* cells which had been transfected with pBluescript lacking the cDNA insert and exposed to IPTG for 0, 1, 3 and 6 h. Lanes E–H contained 1 µg of total RNA of *E. coli* cells which had been transfected with pBluescript containing chick haem oxygenase cDNA insert and similarly exposed to IPTG for 0, 1, 3 and 6 h. Lane I contained 12 mg of total RNA from liver of an untreated (control) chick. Lane J contained 12 µg of total RNA of liver from a chick pretreated with Cd²⁺, and lane K contained 1 µg of poly(A)⁺ RNA of the same pretreated chick liver. The blot was probed with a ³²P-5'-end-labelled synthetic oligonucleotide containing 24 bases, as described in the Materials and methods section.

from rat (Shibahara *et al.*, 1985) and human (Yoshida *et al.*, 1988) sources (Fig. 3). The overall degree of similarity in primary amino acid structure between chick and rat is 62 %, between chick and human is 62 %, and between rat and human is 79 %. Identity is observed for all three proteins between Pro¹²⁹ and Ala¹⁵⁷, suggesting that this region plays a key role in enzymic function. This region includes one histidine residue (at position 135), which, we propose, is important for the binding of the substrate haem to the active centre of the enzyme (Bonkovsky *et al.*, 1990). Currently we cannot exclude the possibility that other conserved histidine residues (at positions 28, 87 and 122)

play such a role. However, other histidine residues that are not fully conserved (e.g. at positions 59, 128, 196 and 249) probably do not. Additional studies, involving site-directed mutagenesis of selected residues, should help clarify this issue.

Of additional interest is the fact that both chick haem oxygenase and rat haem oxygenase-1 contain ten histidine residues, whereas human haem oxygenase-1 contains only six histidine residues (Shibahara *et al.*, 1985; Yoshida *et al.*, 1988). The chick enzyme contains one cysteine residue (at position 110; Fig. 3), in contrast with the rat and human enzymes, which are totally lacking in cysteine.

Fig. 6. Comparison of hydropathy profiles of (a) chick and (b) rat liver haem oxygenase

Relative hydrophobicity is plotted above and hydrophilicity below the '0' line. The numbers on the abscissa refer to the amino acid residues shown in Fig. 3. These profiles were generated by the algorithm of Kyte & Doolittle (1982) in GENEPRO software. The profile for human haem oxygenase-1 is nearly identical.

The degree of similarity of nucleotide sequences in the coding regions between both chick and rat, and chick and human haem oxygenases is 66%, whereas the degree of similarity between rat and human is 81%.

Predicted secondary structures of the three haem oxygenases are also shown schematically in Fig. 3 and are quite similar, with two or three regions of β -sheet, seven of α -helices and five common-turn regions. The rest of the peptides are presumably in random-coil arrangement.

To establish further that the cDNA cloned and sequenced includes the message for haem oxygenase, *E. coli* (XL1-Blue) were transfected with pBluescript with and without the cDNA clone. Growth of the *E. coli* in presence of IPTG for 6 h led to specific and dramatic time-dependent increases in activity of haem oxygenase (no insert, 30; with insert, 4300 pmol of bilirubin/min per mg of protein). The amount of haem oxygenase protein also increased dramatically (Fig. 4). These studies also indicate that post-translational modification dependent upon subcellular organelles found only in eukaryotic cells are not required for production of active haem oxygenase. Other recent studies from our laboratory indicated the importance of phosphatidylcholine for activity of chick haem oxygenase, suggesting that insertion into a lipid bilayer is important for enzyme activity (Bonkovsky *et al.*, 1990). All enzyme activities reported here were measured in the presence of optimal concentrations of phosphatidylcholine and the other components previously described (Bonkovsky *et al.*, 1990).

The molecular mass of the major immunologically reactive protein from transformants (Fig. 4, lanes 5–7) is about 4 kDa greater than that of pure chick haem oxygenase (Fig. 4, lane 1), because the fusion peptide expressed in *E. coli* contains a 36-amino-acid fragment of β -galactosidase. The minor bands of lower molecular mass that increase in amount with time of exposure to IPTG probably represent proteolytic products of the parent fusion peptide.

The amounts of mRNA for chick haem oxygenase expressed in *E. coli* also increased markedly from 0 to 3 h after exposure of the *E. coli* to IPTG (Fig. 5, lanes E–G). There was appreciable breakdown of the full-length message to shorter fragments by three hours and even more after 6 hours of exposure to IPTG (Fig. 5, lanes G and H). This may be due to degradation by RNAase inside *E. coli*. Nevertheless, the amounts of protein and activity of chick haem oxygenase continued to increase (Fig. 4), indicating that the enzyme is rather stable in *E. coli*.

Fig. 5 also shows that total RNA and mRNA from liver of a chick pre-treated with Cd^{2+} , a potent inducer of haem oxygenase (Maines & Kappas, 1974, 1977; Sardana *et al.*, 1980; Lincoln *et al.*, 1988; Bonkovsky *et al.*, 1990), was increased above control (lanes J and K versus lane I), indicating that Cd^{2+} either increases the rate of transcription of, or stabilizes, the message for haem oxygenase in intact chicks. The message that accumulated in liver of cadmium-treated chick was 390–440 bases larger than that corresponding to the cloned cDNA (1.65–1.70 versus 1.26 kb; Fig. 5). Nonetheless, our sequencing result (Figs. 2 and 3) indicated that the full coding region, the stop codon (TGA), plus 68 upstream and 299 downstream nts were cloned. The additional 390–440 bases in the mRNA isolated from liver probably include a poly(A)⁺ tail.

The hydropathy profiles predicted for chick haem oxygenase and rat and human haem oxygenase 1 are very similar (Fig. 6). Probably the membrane-anchor regions are similar, comprising the hydrophobic C-terminal 39 amino acid residues of the proteins (Yoshida & Sato, 1989). A likely candidate site for the action of trypsin to cleave the protein in a lipid bilayer, leaving only the C-terminal 39 residues in the membrane, is the Lys²⁵⁷–Glu²⁵⁸ bond (Fig. 3).

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