Cytosolic ferritin and lipid-associated ferritin are metabolically different in guinea-pig livers

Bill E. CHAM,* H. Peter ROESER and Anne C. NIKLES

Department of Medicine, University of Queensland, Clinical Sciences Building, Royal Brisbane Hospital, Queensland 4029, Australia

A distinct pool of liver ferritin has been described in man, guinea pigs and rats [Cham, Roeser, Nikles & Ridgway (1986) *Clin. Chim. Acta* **158**, 71–79]. This ferritin accounts for approx. 30 % of total intracellular ferritin. It differs from previously described cytosolic and ‘microsomal-fraction’ ferritin by its firm association with lipid and by the absence of heat-stability at 75 °C. The present study demonstrates that cytosolic ferritin and lipid-associated ferritin in guinea-pig livers have distinctly different rates of turnover. Cytosolic ferritin has a rate of turnover approx. 3.5 times as high as lipid-associated ferritin. The apparent metabolic heterogeneity suggests that the two forms of ferritin may have different functional roles.

INTRODUCTION

Ferritin is an iron-containing protein and is present in mammals in the form of organ-specific isoproteins. Studies on the intracellular localization of liver ferritin in rats, with the use of antibody precipitation as a ferritin assay, indicated that the protein was present in approximately equal quantities in cytosol and in light-microsomal fractions (Sargent & Munro, 1975). Ferritin in the microsomal fraction could be made available to antibody either by heating the preparation to 75 °C or by treatment with the anionic detergent sodium deoxycholate (1 %). A separate metabolic role, on the basis of the different cellular localization, has been proposed for these two ferritin pools, but no direct evidence for such differing roles has been adduced.

More recently, a further pool of intracellular ferritin has been identified. This ferritin can be made available to antibody by extraction of liver homogenates with an organic solvent system such as butanol/di-isopropyl ether (Cham & Knowles, 1976; Cham *et al.*, 1986). The non-ionic detergents Triton X-100 and Nonidet P-40 will also release this ferritin from lipid, but it cannot be made available to antibody by treatment with 1 % sodium deoxycholate or by heating to 75 °C (Cham *et al.*, 1988). Thus this ferritin differs from the membrane-associated ferritin described by Sargent & Munro (1975), and it has been called lipid-associated ferritin (LAF). In the liver LAF represents 24–33 % of total cellular ferritin, depending on the species of mammal (Cham *et al.*, 1986). The composition of LAF (ferritin + lipid) is not known. However, ferritin that is released from its conjugate (LAF) by delipidation has a similar molecular size and shows no discernible qualitative differences in electrophoretic mobility or antigenic specificity when compared with cytosolic ferritin (Cham *et al.*, 1986). In addition, the iron content of LAF is similar to that of cytosolic ferritin. In guinea-pig livers the iron-to-protein ratio (by wt.) was 8.6 ± 3.0 % for LAF and 9.2 ± 2.7 % for cytosolic ferritin ($n = 6$, means $\pm$ s.d.) (B. E. Cham, H. P. Roeser & A. C. Nikles, unpublished work). The biological signi-

ficance of the association of ferritin with lipid is not known. The present studies indicate that metabolically LAF behaves differently from cytosolic ferritin.

EXPERIMENTAL

Materials

$\text{Na}_2^{14}\text{CO}_3$ (specific radioactivity 50 mCi/mmol, as aqueous solution) was purchased from Amersham (Australia) Pty. Ltd. (Sydney, N.S.W., Australia). All reagents were of the highest quality commercially available.

Animals

Male white guinea pigs (English short-hair, random outbred) were used in this study. Weanlings were obtained at 2 weeks of age. They were fed *ad libitum* on a semi-synthetic diet [Subcommittee on Laboratory Animals Nutrition (N.R.C.–N.A.S.) (1978)] that had an iron content of 535 ± 18 $\mu\text{g/g}$ (mean $\pm$ s.d.). Ascorbic acid was supplied in the drinking water (600 mg of ascorbic acid/l). On this diet the animals gained approx. 40 g/week in weight (Roeser *et al.*, 1986). At the time that they were killed the weight of the animals was 380 ± 61 g (mean $\pm$ s.d.). Removal of livers was done under diethyl ether anaesthesia. The care of the animals was undertaken in accordance with the guidelines laid down by the National Health and Medical Research Council of Australia.

Incorporation of ^{14}C from $\text{Na}_2^{14}\text{CO}_3$ into liver ferritin

Previous studies had shown that uptake of label into ferritin did not attain a plateau until 2 h after intraperitoneal injection of [^{14}C]leucine (Roeser *et al.*, 1983). Animals received 250 μCi of $\text{Na}_2^{14}\text{CO}_3$ per 100 g body wt. intraperitoneally. The first liver samples were obtained at 3 h after administration of the label. Subsequent samples for determining the rates of synthesis and degradation of ferritin were obtained at 12 and 24 h and at 2, 3, 4, 6 and 8 days. At each time point samples were taken from one to four animals.

Abbreviation used: LAF, lipid-associated ferritin.

* To whom correspondence should be addressed.

Liver preparation

Liver extracts were prepared by homogenizing the liver samples at 4 °C in 4 vol. of phosphate-buffered saline (154 mM-NaCl/0.02 mM-potassium phosphate buffer, pH 7.4) containing 1.54 mM-NaN₃. The homogenates were centrifuged at 30000 *g* for 30 min at 4 °C. The resultant supernatant (which may have possibly lost the iron-rich ferritin by the centrifugation step) was called post-nuclear supernatant (Cham *et al.*, 1986).

Measurement of total liver ferritin, cytosolic ferritin and LAF concentrations

The method employed to separate and measure these three ferritin concentrations in liver tissue has been described elsewhere (Cham *et al.*, 1986). In summary, total liver ferritin concentration was derived by delipidating a sample of post-nuclear supernatant (see below). Ferritin was concentrated by precipitating it with 50 %-saturated (NH₄)₂SO₄ and redissolving it in a small volume of deionized water. This was quantitatively applied to a polyacrylamide-gel slab and, after electrophoresis, the ferritin band was quantitatively excised, eluted and assayed (Roeser *et al.*, 1983; Cham *et al.*, 1985, 1986) (see below). To obtain cytosolic ferritin, a sample of post-nuclear supernatant was heated to 75 °C. The supernatant was then subjected to (NH₄)₂SO₄ precipitation and polyacrylamide-gel electrophoresis, as above. The excised ferritin band was again eluted and assayed. LAF concentration could then be calculated from the difference in these two measurements. Alternatively, LAF was measured directly by quantitatively applying a sample of post-nuclear supernatant to a polyacrylamide-gel slab. On electrophoresis LAF remained at the point of application and the band was quantitatively excised and delipidated. The sample was subjected to further polyacrylamide-gel electrophoresis, and the resultant ferritin band, which co-migrated with cytosolic ferritin and a purified ferritin standard, was quantitatively excised, eluted and assayed. LAF concentrations obtained by this technique were identical with those calculated from the difference between total ferritin and cytosolic ferritin. All ferritin assays were carried out in duplicate.

Delipidation

Lipid was extracted from post-nuclear supernatant or from polyacrylamide-gel eluates by using the biphasic organic solvent system consisting of di-isopropyl ether/butan-1-ol (3:2, v/v) (Cham *et al.*, 1986). This method of delipidation has been shown not to alter the physico-chemical characteristics of proteins (Cham & Knowles, 1976).

Ferritin assay

The protein concentration of ferritin in liver preparations was measured by electroimmunoassay (Carmel & Konijn, 1978). Ferritin for standards was extracted from guinea-pig livers and purified by using the method of Cham *et al.* (1985). Antibody to horse spleen ferritin (Sigma Chemical Co., St. Louis, MO, U.S.A.) was prepared in goats. With each batch of tissue ferritin assays, both guinea-pig liver ferritin and horse spleen ferritin were used (Roeser *et al.*, 1983). The ratio of reactivity of the two ferritins with constant amounts of the antibody was consistent over a 20-fold range of ferritin [determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard] and over a

prolonged time period (horse spleen ferritin/guinea-pig liver ferritin ratio $0.58 \pm 0.07:1$, mean $\pm$ s.d.). This procedure assured consistency of absolute tissue ferritin values between assays (Roeser *et al.*, 1983). Ferritin values quoted are related to the guinea-pig liver ferritin standards. The calibration curve for ferritin was linear over the range 0–300 mg/l. The precision (coefficient of variance 1.5–7 %) of the assay was similar to that described elsewhere (Carmel & Konijn, 1978). The preparation of samples for radioactivity counting was undertaken as previously described (Roeser *et al.*, 1983). Samples were prepared in duplicate, and blank gel slices were also homogenized, eluted and their radioactivities counted for background correction. The instrument used (LKB Rackbeta 1217 liquid-scintillation counter) operated at 40 % efficiency for ¹⁴C. Results were calculated as specific radioactivity (c.p.m./mg of protein) and as tissue radioactivity (c.p.m./total liver ferritin). The iron concentration of ferritin was measured by atomic absorption spectrophotometry as described elsewhere (Roeser *et al.*, 1983).

Ferritin purity

The purity of the isolated cytosolic ferritin and LAF was established by analyses of their protein concentrations by two different procedures. Essentially, a chemical assay was used, with bovine serum albumin as standard (Sedmak & Grossberg, 1977). The chemical assay was based on the quantitative staining of proteins with Coomassie Brilliant Blue G250. Plots of A_{620}/A_{465} absorption ratios were linear over the concentration range 0.5–50 µg for both albumin and ferritin. Traces of polyacrylamide gel did not interfere with the protein assays. The other procedure was an immunological assay (electroimmunoassay), with guinea-pig liver ferritin as standard (Carmel & Konijn, 1978). The guinea-pig liver ferritin standard was standardized by the chemical procedure, with bovine serum albumin as standard.

Comparison of immunological reactivity of cytosolic ferritin with LAF

Cytosolic ferritin and LAF were measured by electroimmunoassay, with guinea-pig liver ferritin as standard (Carmel & Konijn, 1978), and by chemical assay, with bovine serum albumin as standard (Sedmak & Grossberg, 1977). The guinea-pig liver ferritin was standardized by the chemical procedure, with bovine serum albumin as standard. If the ferritins in the two compartments have equal immunological reactivity, then the cytosolic ferritin/LAF ratio by the two different protein assays would be 1:1.

Calculation of rates of synthesis and catabolism

Under steady-state conditions for first-order kinetics the rate of fall in specific radioactivity (c.p.m./mg of protein) measures the rate of protein synthesis, whereas the rate of loss of total radioactivity in a tissue or tissue compartment measures the catabolic rate (Millward, 1970). Thus:

$$k_s = \frac{1}{t} \cdot \ln \left(\frac{S_0}{S_t} \right)$$

and

$$k_c = \frac{1}{t} \cdot \ln \left(\frac{C_0}{C_t} \right)$$

where k_s and k_c are first-order rate constants for synthesis and catabolism respectively, S_0 and S_t are specific

Table 1. Comparison of immunological reactivity of cytosolic liver ferritin with lipid-associated liver ferritin

Cytosolic ferritin and LAF concentrations of guinea-pig liver homogenates were measured by the electroimmunoassay procedure and by the Coomassie-Blue-assay procedure. The ratios of the obtained concentrations were then calculated.

	Concentration ($\mu\text{g/ml}$)		Electro-immunoassay/ Coomassie Blue assay ratio
	Electro-immunoassay	Coomassie Blue assay	
Cytosolic ferritin	11.4	11.4	1.00
	14.2	17.3	0.82
	9.4	9.3	1.01
	10.3	10.1	1.02
	10.4	11.2	0.93
	10.0	7.4	1.35
	11.3	9.3	1.21
	13.0	9.3	1.40
Mean $\pm$ S.D. ...	11.3 $\pm$ 1.6*	10.7 $\pm$ 3.0*	1.10 $\pm$ 0.21
LAF	16.2	13.4	1.21
	6.4	7.9	0.81
	38.2	37.0	1.03
	5.4	5.1	1.06
	26.8	37.6	0.71
	14.8	9.4	1.57
	8.1	10.2	0.79
Mean $\pm$ S.D. ...	16.6 $\pm$ 12.1**	17.2 $\pm$ 13.9**	1.03 $\pm$ 0.30

* and ** Not significantly different ($P \geq 0.05$). The immunological reactivity of cytosolic ferritin and LAF was similar and was calculated by:

$$\left(\frac{\text{Electroimmunoassay cytosolic ferritin}}{\text{Coomassie-Blue-assay cytosolic ferritin}} \right) / \left(\frac{\text{Electroimmunoassay LAF}}{\text{Coomassie-Blue-assay LAF}} \right) = 1.07 \pm 0.19 \text{ (mean } \pm \text{ S.D.)}$$

radioactivities at times 0 and t , and C_0 and C_t are total radioactivities at times 0 and t . If $\ln C$ or $\ln S$ is plotted against time, $t_{1/2}$ can be derived and k calculated from $k = \ln 2/t_{1/2}$.

The data were analysed with a Stat Pack V4 program (Western Michigan University) for fitting multiple linear-regression equations and analysis of variance. Absolute rates of ferritin synthesis were calculated from the product of the rate constant for synthesis and the total liver ferritin pool.

RESULTS AND DISCUSSION

The ferritin concentration in the delipidated post-nuclear supernatant, which represented the total ferritin pool, was $457 \pm 256 \mu\text{g/g}$ wet wt. of liver (mean $\pm$ S.D., $n = 22$). Cytosolic ferritin and LAF concentrations were 316 ± 158 and $140 \pm 108 \mu\text{g/g}$ wet wt. of liver (means $\pm$ S.D.) respectively. Thus LAF represented about 30% of the total ferritin pool. Quantities of cytosolic ferritin and LAF, expressed as μg of ferritin in total liver, were 5017 ± 2872 and 2270 ± 1888 (means $\pm$ S.D.) respectively. LAF concentrations obtained by the two methods described were similar (paired t test, $t = 1.331$, degrees of freedom = 16, $P =$ not significant). LAF values obtained by the two methods were pooled for subsequent calculations. Ferritin concentrations did not vary significantly in groups of animals killed over the 8 days of the experiment.

When assayed by immunological and chemical procedures, both cytosolic ferritin and LAF gave similar results, indicating that the isolated cytosolic ferritin and LAF were pure (Table 1).

The immunological reactivity of ferritins in the two compartments was similar (Table 1). Because of this, it was possible to calculate the specific radioactivity for ferritin in each compartment.

The decline with time in specific radioactivity of cytosolic ferritin and of LAF represented the rate of dilution of labelled ferritin by newly formed unlabelled protein. The calculated regression lines yielded $t_{1/2}$ values of 1.36 ± 0.19 days (mean $\pm$ S.E.M.) for cytosolic ferritin and 4.56 ± 3.31 days (mean $\pm$ S.E.M.) for LAF. The decline with time of total liver ferritin radioactivity represented catabolism of labelled ferritin. The $t_{1/2}$ value for cytosolic ferritin was 1.45 ± 0.30 days and that for LAF was 5.47 ± 3.66 days (means $\pm$ S.E.M.). Table 2 sets out the corresponding rate constants and absolute rates of ferritin synthesis, calculated from total liver ferritin pool sizes. The synthetic rate was similar to the catabolic rate in the cytosolic ferritin pool. Similarly, synthesis was similar to degradation for the LAF pool. This confirmed that a steady state prevailed throughout the study.

Previous measurements of liver ferritin turnover have utilized the technique of heating liver homogenates to 75°C in order to coagulate most cellular proteins, followed by assay of ferritin in the supernatant (Drysdale & Munro, 1966; Glass & Doyle, 1972). The results obtained therefore apply only to heat-stable ferritin, equivalent to the cytosolic ferritin in the present study. Values obtained for catabolic rates ($t_{1/2}$) for liver ferritin in rats (Drysdale & Munro, 1966; Glass & Doyle, 1972) and guinea pigs (Roeser, 1983) have varied from 1.3 to 3.6 days. Much of this variation can be attributed to the method used for labelling ferritin. [^3H]Leucine is sub-

Table 2. Synthesis and catabolism of cytosolic liver ferritin and lipid-associated liver ferritin

Animals received 250 μCi of $\text{Na}_2^{14}\text{CO}_3$ per 100 g body wt. intraperitoneally. Liver samples were obtained at various times (3 h to 8 days) after administration of the label. The decay of specific radioactivity and the decay of total radioactivity of cytosolic ferritin and LAF were used to calculate their synthetic and catabolic rates respectively. k_s is the rate constant for ferritin synthesis, and k_c is the rate constant for ferritin catabolism, and the values are means $\pm$ S.E.M. Ferritin synthesis was calculated from total liver ferritin pool sizes, and mean values only are given, as k_s values for individual animals cannot be derived.

	Cytosolic ferritin	LAF	P
k_s (days $^{-1}$)	0.51 ± 0.07	0.15 ± 0.11	< 0.02
Synthesis (mg/day)	2.56	0.34	
k_c (days $^{-1}$)	0.48 ± 0.10	0.13 ± 0.09	< 0.02

stantially re-utilized, whereas $\text{Na}_2^{14}\text{CO}_3$ or [*guanidino*- ^{14}C]arginine is not (Millward, 1970; Glass & Doyle, 1972). Direct comparisons of two techniques of labelling ferritin in two centres have yielded $t_{1/2}$ values of 3.6 (leucine) and 1.3 (arginine) days (Glass & Doyle, 1972) and 3.0 (leucine) and 2.1 (arginine) days (Munro & Linder, 1978). It is likely that the lower values represent the true catabolic rate, and the $t_{1/2}$ value of 1.45 days obtained for guinea-pig liver cytosolic ferritin in the present study is in accord with these results. In contrast, the newly described pool of LAF was found to have a much lower rate of turnover, as determined by both the rate of synthesis and the rate of catabolism. The significance of this observation in terms of the function of these two pools of intracellular ferritin remains unclear.

Structural variability in ferritin occurs not only between animal species, but between organ systems in one animal, within cells of one type of tissue, and even between similar cell systems at times of differing functional states (Van Wyck *et al.*, 1971; Crichton *et al.*, 1973; Yamada & Gabuzda, 1974; Brown & Theil, 1978; Linder *et al.*, 1981; Stefanini *et al.*, 1982; Mertz & Theil, 1983; Ihara *et al.*, 1984; Theil, 1987).

The one known function of cellular ferritin is to serve as a repository of iron in a soluble, accessible, non-toxic form. However, within this one fundamental role, at least three subsidiary functions have been identified: one, shared by all cells, is the provision of iron for numerous iron-dependent cellular metabolic activities (e.g. respiration); a second is to act as a reservoir (e.g. in hepatic parenchymal cells) to meet potential large needs of iron by other tissues, such as the bone marrow; a third is to act as a sink designed to trap potentially toxic forms of iron that might be generated during conditions of stress (e.g. inflammatory states) (Theil, 1987).

This structural or functional heterogeneity of ferritin has not hitherto been linked to any difference in metabolic characteristics, apart from the observation that acute changes in the iron status of rats (induced by iron administration or venesection) can alter the rate of both

synthesis and catabolism of liver ferritin (Drysedale & Munro, 1966; Munro & Linder, 1978).

In contrast, we have identified a metabolically distinct pool of ferritin, comprising some 30% of total cellular ferritin, whose functional role has yet to be determined. However, if the structural association of this ferritin with lipid has any functional implications, it would seem likely that it acts as a sink for ionic iron in proximity to lipid membranes. This activity would serve to diminish iron-catalysed generation of oxygen free radicals and consequential lipid degradation (Thomas & Aust, 1986). Such a role for LAF is unproven, but the concept could serve as a working hypothesis for further study.

We thank Mrs. K. Ridgway for technical assistance and Mr. B. Maher of Prentice Computer Centre for assistance with the data analyses. A.C.N. is supported by the National Health and Medical Research Council of Australia.

REFERENCES

- Brown, J. E. & Theil, E. C. (1978) *J. Biol. Chem.* **253**, 2673–2678
- Carmel, N. & Konijn, A. M. (1978) *Anal. Biochem.* **85**, 499–505
- Cham, B. E. & Knowles, B. R. (1976) *J. Lipid Res.* **17**, 176–181
- Cham, B. E., Roeser, H. P., Nikles, A. C. & Ridgway, K. (1985) *Anal. Biochem.* **151**, 561–565
- Cham, B. E., Roeser, H. P., Nikles, A. C. & Ridgway, K. M. (1986) *Clin. Chim. Acta* **158**, 71–79
- Cham, B. E., Roeser, H. P. & Nikles, A. C. (1988) *Clin. Chem.* **34**, 152–154
- Crichton, R. R., Millar, J. A., Cumming, R. L. C. & Bryce, C. F. A. (1973) *Biochem. J.* **131**, 51–59
- Drysedale, J. W. & Munro, H. N. (1966) *J. Biol. Chem.* **241**, 3630–3637
- Glass, R. D. & Doyle, D. (1972) *J. Biol. Chem.* **247**, 5234–5242
- Ihara, K., Maeguchi, K., Young, C. T. & Theil, E. C. (1984) *J. Biol. Chem.* **259**, 278–283
- Linder, M. C., Nagel, G. M., Roboz, M. & Hungerford, D. M., Jr. (1981) *J. Biol. Chem.* **256**, 9104–9110
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mertz, J. R. & Theil, C. C. (1983) *J. Biol. Chem.* **258**, 11719–11726
- Millward, D. J. (1970) *Clin. Sci.* **39**, 577–590
- Munro, H. N. & Linder, M. C. (1978) *Physiol. Rev.* **58**, 317–396
- Roeser, H. P. (1983) *Semin. Hematol.* **20**, 91–100
- Roeser, H. P., Sizemore, D. J., Nikles, A. C. & Cham, B. E. (1983) *Br. J. Haematol.* **55**, 325–333
- Roeser, H. P., Cham, B. E., Nikles, A. C. & Ridgway, K. (1986) *Vitaminologia* **2**, 101–108
- Sargent, K. S. & Munro, H. N. (1975) *Exp. Cell Res.* **93**, 15–22
- Sedmak, J. J. & Grossberg, S. E. (1977) *Anal. Biochem.* **79**, 544–552
- Stefanini, S., Chiaccone, E., Arosio, P., Finazzi-Agrò, A. & Antonini, E. (1982) *Biochemistry* **21**, 2293–2299
- Subcommittee on Laboratory Animals Nutrition (N.R.C.–N.A.S.) (1978) *Nutrient Requirements of Laboratory Animals*, pp. 59–69, National Research Council, Washington
- Theil, E. C. (1987) *Annu. Rev. Biochem.* **56**, 289–315
- Thomas, C. E. & Aust, S. D. (1986) *J. Biol. Chem.* **261**, 13064–13070
- Van Wyck, C. P., Linder-Horowitz, M. & Munro, H. N. (1971) *J. Biol. Chem.* **246**, 1025–1031
- Yamada, H. & Gabuzda, T. G. (1974) *J. Lab. Med.* **83**, 477–488