TURNOVER OF HEME AND PROTEIN MOIETIES OF RAT LIVER MICROSOMAL CYTOCHROME b_5*

K. W. Bock** and P. Siekevitz

Department of Cell Biology
The Rockefeller University
New York, N. Y. 10021

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SUMMARY. Turnover studies on purified rat liver microsomal cytochrome b_5, using a double-labeling procedure with ^{14}C-guanidino-L-arginine and ^{3}H-δ-amino-levulinic acid, gave half-life values of ~ 3.5 and of ~ 1.7 days for the protein and heme moieties, respectively. Results of pulse experiments and the effects of cycloheximide point to the existence of heme and cytochrome b_5 apoprotein pools.

Recent studies from this and other laboratories have shown that, in rat liver endoplasmic reticulum membranes, total protein and lipids are turning over asynchronously (1) and that individual proteins are turning over at different rates (1-4). However, apparently conflicting results have been published concerning the turnover of the heme and protein moieties of one of the above proteins, cytochrome b_5. Garner and McLean (5), in studies with livers of cycloheximide-treated rats, concluded that heme incorporation into total microsomes and into cyt. b_5 is independent of protein synthesis. However, Druyan *et al.* (6), using separate animals for the heme and protein studies, found the half-lives for the heme and protein moieties of this cytochrome to be the same But their half-life value for the protein moiety of cyt. b_5 was in variance with the one reported by Kuriyama *et al.* (2), using the same amino acid precursor as label. The half-life value reported by Druyan *et al.* (6) for the heme of cyt. b_5 agrees with the value reported by Greim *et al.* (7). In the following, the heme and protein turnovers of microsomal cyt. b_5 has been reinvestigated by double labeling *in vivo* the same animals with (2,3-^{3}H)-δ-aminolevulinic acid (δ-Ala) for the heme and ^{14}C-guanidino-L-arginine for the protein moieties.

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METHODS. Male Sprague-Dawley rats (180-200 g) fed ad libidum a standard diet (Lab Chow, Ralston Purina Co.) and fasted overnight (20 hr) before sacrifice were used throughout the experiments. Microsomal cyt. b_5 was solubilized by trypsin digestion and purified to a value of 60-70 nmoles/mg protein as described by Omura et al. (1) except that the microsomes were washed with 0.15 M KCl + 10 mM EDTA (2) prior to digestion. From the trypsin digestion residue, a fraction containing the cyt. P-450 converted to cyt. P-420, ("CO-binding particles" (8)) was prepared by steapsin digestion according to Omura and Sato (8). Specific ^{14}C radioactivities of the cytochrome b_5 protein and of the proteins in the CO-binding particles were determined on TCA-precipitated aliquots, as given by Omura et al. (1) for purified cyt. b_5 and total microsomal proteins. The amount of heme of purified cyt. b_5 was determined by the dithionite-reduced minus oxidized difference spectrum according to Omura and Sato (8), and its ^3H radioactivity was determined directly on a non-precipitated aliquot. To insure that all the ^3H counts in the cyt. b_5 were attributable to heme radioactivity, the heme was extracted by the procedures given below; it was found that the specific activity of the extracted heme (based on pyridine hemochromogen) was approximately the same as that of the unextracted cyt. b_5. The extraction was carried out by treating the cyt. b_5 for ~ 0.5 hr with 2 vol. of 2-butanone-0.1 M HCl (9). When the radioactivity of the heme in the CO-binding particles was determined, the fraction was first extracted with 9 vol. ice-cold acetone, then treated as above or with 4 vol. cold acetone-0.2 M HCl (10) followed by extraction with 4 vol. hexane (7). In all cases, the organic phase was carefully evaporated and the hemes were determined as pyridine hemochromogen as described by Omura and Sato (8); the radioactivity was measured in this pyridine solution. Radioactivity was determined by liquid scintillation counting in Bray's solution (11) in a Mark I Nuclear Chicago Co. counter. In the channels used, the ^{14}C window contained virtually no ^3H counts while the ^3H window had 20% ^{14}C counts. Efficiencies were 35% for ^{14}C and 13-19% for ^3H. Counting was carried out to at least 5% accuracy. Quenching was monitored by a channel-ratio technique and by the addition of an internal standard.

In all cases given in the Tables, the specific heme radioactivity of the cyt. b_5 was calculated by counting directly the purified enzyme and determining the amount of heme by its difference spectra. The specific radioactivity of the heme in the CO-binding particles was calculated by counting the extracted heme and determining its amount by the pyridine hemochromogen method.

RESULTS AND DISCUSSION. In Table I are given the results of a kinetic experiment on heme labeling in various microsomal fractions after an *in vivo* injection of the precursor ^{3}H-*d*-Ala (12). Trypsin digestion of microsomes solubilizes ~ 70% of the cyt. b_5 (1); the heme specific radioactivity of the trypsin extractable hemoprotein remains constant for 2 to 24 hr post injection. Steapsin treatment of the trypsin digestion residue solubilizes the rest of the cyt. b_5, leaving behind a sediment with cyt. P-420 as the only detectable cytochrome (8). The heme specific radioactivity of the steapsin extractable hemoprotein remains constant while that of the heme in the steapsin digestion residue decreases from

TABLE I

Time Course of Heme Incorporation into Microsomal Cyt. b_5

Noe rats (180 g) were injected intravenously with 10 μc (2,3-^{3}H)-*d*-Ala (25 c/mmole) per rat and 3 rats were sacrificed at each time point after labeling. Pooled liver microsomes were first treated with trypsin (1) and the cyt. b_5 purified from the trypsin extract (1). The residue of trypsin digestion (containing ~ 30% of the original microsomal cyt. b_5) was then treated with steapsin (8) to release the remainder of the cyt. b_5, leaving the CO-binding cytochrome in the final residue. For the trypsin extract and for purified cyt. b_5, the heme was not extracted; for the steapsin extract and the steapsin residue, it was extracted as given in Methods.

Time after labeling (hr)	Trypsin extract	Steapsin extract	Purified cyt. b_5	Steapsin residue (CO-binding particles)
	(dpm/nmole heme)			
2	3110	2560	890	7300
8	3180	2580	1230	6100
24	3060	2640	1920	5600

the 2-hr value. However, the heme specific radioactivity of the cyt. $\underline{b}_5$ purified from the trypsin extract is only 30% of that found in this extract, at the two hr time point. A gradual increase in specific radioactivity is observed, which eventually approaches (at 24 hr) the heme specific radioactivities found for the total trypsin extractable hemoprotein. Because of this gradual incorporation, we have to assume that heme exchange is taking place between cyt. $\underline{b}_5$ and a heme pool which is not susceptible to the rapid degradation of excess heme described by Tenhunen *et al.* (13). This pool could be very small since the measured radioactivity only represents $> 10^{-4}$ nmoles heme. These observations also suggest that it is necessary to isolate a pure cytochrome to obtain reliable kinetic data, particularly at short times after a pulse. The nature of the compound responsible for the seemingly spurious radioactivity in the trypsin extract at the early time points is unknown, but its extractibility by heme solvents suggests that it could be a non-specifically bound heme.

The effect of cycloheximide on a two-hour pulse labeling of the heme of purified cyt. $\underline{b}_5$ was next studied; the inhibitions of protein synthesis and of heme synthesis and incorporation were measured by simultaneous injections of ^{14}C-L-

TABLE II

Influence of Cycloheximide on Heme and Leucine Incorporation into Microsomal Cyt. $\underline{b}_5$

Three rats (180 g) received intraperitoneally 5 mg/kg body weight cycloheximide. One hour later they, together with 3 control rats, were injected intravenously with a mixture of 10 μc (2,3-^{3}H-)-δ-Ala (25 c/mmole) and 20 μc ^{14}C-U-L-leucine (273 mc/mmole) per rat. The animals were sacrificed 2 hr after labeling and the livers of treated and of control rats were separately pooled. Procedures for the purification of microsomal cyt. $\underline{b}_5$ and the measurement of specific heme and protein radioactivities are given under Methods.

Fraction	Cycloheximide treatment	^{3}H dpm/nmole heme	^{14}C dpm/mg protein
Cyt. $\underline{b}_5$	-	1100	1670
	+	450	135

leucine and of ^{3}H-δ-Ala. As shown in Table II, a 3-hr treatment with cycloheximide inhibited leucine incorporation into purified cyt. b_5 protein by nearly 90% in a 2-hr labeling experiment. At the same time it inhibited labeling of the heme of this cytochrome by 60%. After a one-hour pulse, Garner and McLean (5) found heme labeling in cyt. b_5 to be unaffected when protein synthesis was almost completely inhibited by cycloheximide. This inhibitor was also used by Felicetti et al. (14) to demonstrate a disassociation between heme and globin synthesis in the case of hemoglobin. The discrepancy between the inhibitions of protein synthesis and of heme incorporation into purified cyt. b_5 could be explained by heme incorporation into prexisting cyt. b_5 apoprotein or by heme exchange on the cyt. b_5 molecule. Recently Negishi and Omura (15) and Hara and Minakami (16) have presented evidence for the occurrence of cyt. b_5 apoprotein in liver microsomes.

To decide whether heme exchange is occurring in vivo the turnovers of the heme and protein moieties of cyt. b_5 were measured with a double labeling procedure in which the heme was specifically labeled with ^{3}H-δ-Ala and the protein moiety with ^{14}C-guanidino-L-arginine, a protein label with minimal reutilisation in liver (17). As is shown in Fig. 1, the half-lives thus obtained for the heme ($\sim$ 1.7 days) and for the apoprotein ($\sim$ 3.5 days) of microsomal cyt. b_5 differ markedly from one another. The half-lives for the protein of cyt. b_5 and for the mixed protein of the steapsin digestion residue ($\sim$ 2.5 days) are in agreement with values previously reported by Kuriyama et al. (2) for purified cyt. b_5 and total microsomal proteins respectively, using the same label. But our $\sim$ 3.5 day value for the half life of the cyt. b_5 protein differs from the $\sim$ 2.5 day value obtained by Druyan et al. (6), while our value for the half-life of the heme of cyt. b_5 is slightly shorter than the 2.3 days of the purified cyt. b_5 reported by the same authors (6), and the 1.9 days given by Greim et al. (7) for hemes extracted from the supernatant of steapsin digested microsomes. A difference between the half lives of the protein ($\sim$ 2.5 days, as measured with ^{14}C-guanidino-labeled arginine) and of the heme moieties ($\sim$ 1.8 days, as measured with δ-Ala) was found by Poole et al. (18) for the catalase of liver peroxisomes. They concluded,

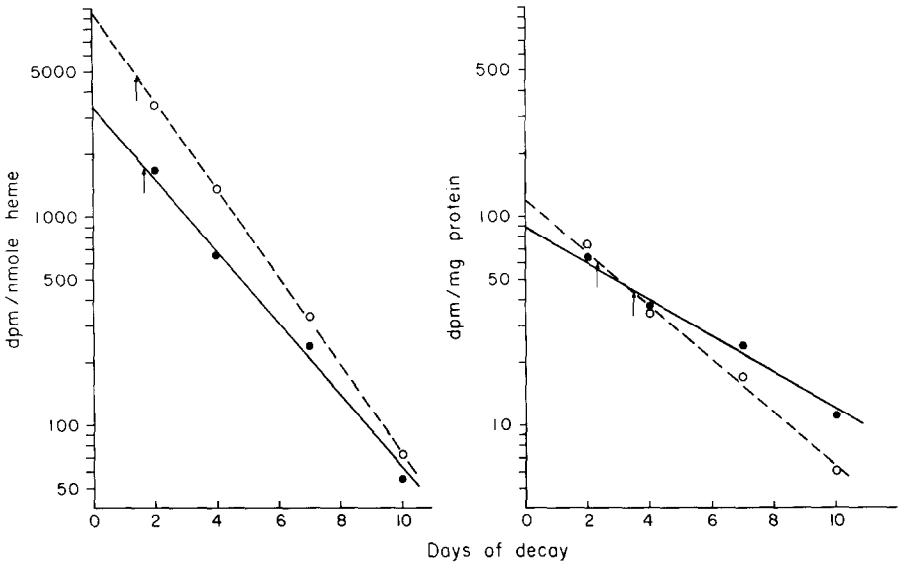

Fig. 1. Turnovers of heme and protein of microsomal cyt. b_5. Twelve rats (200 g) were injected intravenously with a mixture of 10 μc (2,3-^{3}H)-δ-Ala (25 c/mmole) and 25 μc ^{14}C-guanidino-L-arginine (5 mc/mmole) per rat. Three rats were sacrificed 2,4,7 and 10 days after labeling, and the livers pooled at each time point. Procedures for the preparation of the fractions and measurement of specific heme and protein radioactivities are given under Methods. •—•, microsomal cyt. b_5; o—o, extracted hemes and mixed proteins from CO-binding particles. Vertical arrows indicate half-lives.

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however, that in that case the heme and apoprotein probably turn over in synchrony, because they felt that their data were not free of statistical uncertainty and that label reutilization is still possible even for guanidino-labeled arginine. Our finding of a greater difference in the turnovers of the heme and protein moieties indicate to us that in the case of cyt. b_5 the turnover of membrane components is asynchronous down to the level of an apoprotein and its prosthetic group.

Our turnover data also suggest that a slow heme exchange occurs on the cyt. b_5 while the molecule is bound to the endoplasmic reticulum membranes. Heme exchange among hemoglobin molecules has previously been reported (19).

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