Iodothyronine 5'-deiodinase activity as an early event of prenatal brown-fat differentiation in bovine development

Marta GIRALT,* Louis CASTEILLA,†‡ Octavi VIÑAS,* Teresa MAMPEL,* Roser IGLESIAS,* Jacques ROBÉLIN† and Francesc VILLARROYA*§

*Unitat de Bioquímica i Biologia Molecular B, Departament de Bioquímica i Fisiologia, Facultat de Biologia, Universitat de Barcelona, Diagonal 645, 08071-Barcelona, Spain, †Laboratoire de Production de Viande, INRA, 63122-Theix, France, and ‡Centre de Recherches sur la Nutrition, Centre National de la Recherche Scientifique, 9 rue Jules Hetzel, 92190-Meudon-Bellevue, France

Iodothyronine 5'-deiodinase activity appears to be a type I enzyme in bovine brown adipose tissue, on the basis of its high K_m for 3,3',5'-tri-iodothyronine ('reverse T_3 ') (in the micromolar range) and sensitivity to propylthiouracil inhibition. This enzyme activity is already detectable in perirenal adipose tissue of bovine fetuses in the second month of gestation, reaches peak values around the seventh month of fetal life, declines before birth, becomes lower after parturition and finally undetectable in the adult cow. Iodothyronine 5'-deiodinase activity is present in the pericardic, peritoneal and intermuscular adipose depots of the neonatal calf, but it is always undetectable in the subcutaneous adipose tissue. It is concluded that iodothyronine 5'-deiodinase is a specific feature of brown fat in the bovine species that is not shared by white adipose tissue. Peak values of 5'-deiodinating activity appear as an early event in the prenatal differentiation programme of bovine brown-fat cells as they occur when uncoupling-protein-gene expression first starts.

INTRODUCTION

Brown fat has long been recognized as a tissue whose main function is non-shivering thermogenesis (Nicholls *et al.*, 1986). The thermogenic capacity of brown fat is due to the occurrence of the 'uncoupling protein', a component unique to the brown-adipose-tissue mitochondria.

More recently, the presence of an important type II iodothyronine 5'-deiodinase activity in rat brown adipose tissue has been reported (Leonard *et al.*, 1983). Iodothyronine 5'-deiodinase is responsible for thyroxine (T_4) deiodination to tri-iodothyronine (T_3), and the available data based on studies in the rat point to the existence of at least two different enzymic forms (Larsen *et al.*, 1981). Type I enzyme is mainly present in liver and kidney, has K_m values for T_4 and 'reverse T_3 ' (rT_3 ; 3,3',5'-tri-iodothyronine) in the micromolar range and is inhibited uncompetitively by propylthiouracil (PTU) (Visser, 1979; Leonard & Rosenberg, 1980). Type II enzyme has been found in the brain, pituitary and brown fat, it has a K_m for T_4 and rT_3 in the nanomolar range, and it is not inhibited by propylthiouracil (Visser *et al.*, 1982, 1983; Leonard *et al.*, 1983), at least in the presence of high (millimolar range) dithiothreitol concentrations (Goswami & Rosenberg, 1986).

The functional role of iodothyronine 5'-deiodinase activity in rat brown fat seems to have two different aspects. First, brown adipose tissue is a substantial source of systemic T_3 in the rat, at least in situations of high activity of the tissue (Fernández *et al.*, 1987). Secondly, the correlation noted between the levels of brown-fat thermogenesis and iodothyronine 5'-deiodinase activity in different physiological or experimental situations of adult rodents (Silva & Larsen,

1983; Kates & Himms-Hagen, 1985; Giralt *et al.*, 1986; Bianco & Silva, 1987) have suggested that locally produced T_3 may be involved in the thermogenic activity of the tissue.

Brown fat occurs not only in small mammals, but also in larger ones, such as ruminants (Alexander *et al.*, 1975) or primates (Strielemann *et al.*, 1985), including humans (Lean & James, 1986), but the physiological function and biochemical characterization of brown adipose tissue in these species have particular aspects. The main occurrence of brown fat in the bovine and ovine species occurs prenatally and immediately after birth, and it is followed by a postnatal involution of the brown appearance and a rapid replacement for white fat (Alexander *et al.*, 1975; Gemmel & Alexander, 1978; Casteilla *et al.*, 1987). The overall recruitment of the thermogenic apparatus of brown fat, including the induction of uncoupling-protein-gene expression, develops prenatally either in rats (Ricquier *et al.*, 1986) and ruminants (Casteilla *et al.*, 1987). Recently, the bovine species has appeared as a specially interesting model to study the prenatal mechanisms for the recruitment of the thermogenic apparatus of brown fat as well as the relationships between brown and white adipose tissues (Casteilla *et al.*, 1987, 1989). In the present work we have studied the occurrence of iodothyronine 5'-deiodinase activity in bovine brown fat as well as its changes throughout the fetal and neonatal life in order to gain insight in the role of this enzyme activity in the prenatal development of functional brown fat and its postnatal involution.

MATERIALS AND METHODS

Animals and tissue sampling

Bovine (Friesian) fetuses were obtained after slaughter

Abbreviations used: T_3 , 3,3',5'-tri-iodothyronine; T_4 , tetraiodothyronine (thyroxine); rT_3 , 'reverse T_3 ' (3,3',5'-tri-iodothyronine); DTT, dithiothreitol; PTU, propylthiouracil.

§ To whom correspondence and reprint requests should be sent.

of dairy cows, and samples of adipose tissue were collected from the perirenal site. Values for gestational age were deduced from the date of dairy-cow insemination. When postnatal adipose tissue was studied, samples were obtained from the perirenal, pericardic, mesenteric, peritoneal, retroperitoneal, intermuscular and subcutaneous sites. Samples of liver and muscle from newborn calves, as well as of perirenal adipose tissue from an adult cow, were also obtained. All samples were dissected and immediately frozen in liquid N₂ and stored at -80 °C until further processing. Samples studied here are fragments of the same tissue samples already used in our previous study on the mitochondrial protein mRNA expression during bovine brown-fat development (Casteilla *et al.*, 1989).

Iodothyronine 5'-deiodinase assay

Outer-ring(5')-deiodinating activity was assayed by quantifying the ¹²⁵I liberated from L-[5'-¹²⁵I]rT₃, as described previously (Leonard & Rosenberg, 1980). The standard conditions for the bovine adipose-tissue iodothyronine 5'-deiodinase assays were established from the characterization studies.

Fragments of frozen tissue were weighed and homogenized in ice-cold 0.32 M-sucrose/10 mM-Hepes/100 mM-dithiothreitol (DTT), pH 7.0. Homogenates were centrifuged at 80 g for 10 min, fat supernatants were discarded and the infranatants were used for iodothyronine 5'-deiodinase assays. The protein content of the infranatant was determined by the method of Zak & Cohen (1961). Standard assays were performed at final concentrations of 2 µM-rT₃, 0.2 nM purified L-[5'-¹²⁵I]rT₃ (5000 c.p.m./fmol), 100 mM-DTT, 0.1 M-potassium phosphate buffer, pH 7.0, 1 mM-EDTA. Incubations were performed at 37 °C in a final volume of 100 µl containing 150–200 µg of infranatant protein. Reactions were stopped 5 min later by adding 50 µl of ice-cold calf-serum/10 mM-propylthiouracil (1:1) and 350 µl of 10% (w/v) trichloroacetic acid. Separation of iodine from iodothyronines was done by ion-exchange chromatography on a Dowex 50W X2 column (Bio-Rad) equilibrated in 10% (v/v) acetic acid (Leonard & Rosenberg, 1980). Assays were performed in triplicate. Non-enzymic I⁻ production was estimated by incubation of free tissue medium and used as a blank control. High-specific-radioactivity L-[5'-¹²⁵I]rT₃ was obtained by radioiodination with ¹²⁵I⁻ (IMS 30; Amersham International) using 3,3'-di-iodothyronine as substrate (Kochupillai & Yallow, 1978).

For the characterization of bovine brown-adipose-tissue iodothyronine 5'-deiodinase activity, pooled samples of perirenal adipose tissue from fetuses at days 184, 193, 211, 213 and 234 of gestation were used, as well as pooled samples of liver from 0-, 2- and 3-day-old bovine newborns for comparative purposes. The iodothyronine 5'-deiodination activity was assessed as a function of incubation time and of substrate (0.5–8 µM-rT₃) and co-substrate (5–150 mM-DTT) concentrations. The ability of 1 mM-PTU to inhibit the enzyme activity was studied at different concentrations of DTT (1 mM, 10 mM and 150 mM) and rT₃ (2 nM, 0.5 µM and 2 µM), as well as the inhibitory action of lower PTU concentrations (0.1 mM).

RESULTS

Characterization of iodothyronine 5'-deiodinase activity in bovine perirenal adipose tissue

Assays of liver as well as perirenal-brown-adipose-tissue iodothyronine 5'-deiodinase activity at different incubation times showed that linearity could only be guaranteed during the first 5 min of incubation at the different rT₃ and DTT concentrations used for the kinetic-characterization studies and under the final conditions established to be optimal for the standard enzymic assay. Therefore all the assays of enzyme activity were performed with an incubation time of 5 min.

Double-reciprocal plots of liver and perirenal-brown-fat iodothyronine 5'-deiodinating activity in the presence of increasing DTT concentrations and a high unvarying substrate concentration (2 µM-rT₃) resulted in an apparent Michaelis constant (*K_m*) for DTT in the millimolar range in both tissues, as shown in Table 1. When the effect of varying substrate (rT₃) concentration on the 5'-deiodinating activity was studied in the presence of a high co-substrate concentration (150 mM-DTT), the resulting double-reciprocal plots for liver and brown fat resulted in an apparent *K_m* of the iodothyronine 5'-deiodinase activity for rT₃ in the micromolar range (see Table 1).

When the inhibitory effect of 1 mM-PTU on the iodothyronine 5'-deiodinating activity was studied, assays performed at high substrate (2 µM-rT₃) concentrations resulted in 97 and 90% inhibition of the enzymic activity in liver and brown-fat samples respectively in the presence of 150 mM-DTT. A 97 and 99% inhibition was found for both tissues respectively in the presence of 10 mM-DTT, and a 90 and 92% inhibition in

Table 1. Kinetic characteristics of iodothyronine 5'-deiodinase activity in bovine liver and perirenal adipose tissue

Results were obtained from double-reciprocal plots of iodothyronine 5'-deiodinase activity in bovine liver (pooled samples of 0-, 2- and 3-day-old animals) and perirenal adipose tissue (pooled samples of fetuses on days 184, 193, 211, 213 and 234 of gestation). Assays were performed as a function of DTT (5–150 mM) concentration in the presence of a high unvarying rT₃ concentration and as a function of rT₃ (0.5–8 µM) concentration in the presence of a high unvarying DTT concentration.

	Unvarying rT ₃ (2 µM)		Unvarying DTT (150 mM)	
	<i>V_{max}</i> , (nmol of I ⁻ /h per mg of protein)	<i>K_m</i> for DTT (mM)	<i>V_{max}</i> , (nmol of I ⁻ /h per mg of protein)	<i>K_m</i> for rT ₃ (µM)
Liver	26.3	2.7	80.1	14.0
Perirenal adipose tissue	3.2	6.3	4.3	2.6

Fig. 1. Iodothyronine 5'-deiodinase activity in bovine perirenal adipose tissue during fetal and postnatal life

Individual points are the means of triplicate determinations of samples corresponding to individual fetuses or newborns. The arrow indicates the fetal age when the mRNA coding for the uncoupling protein first appears (Casteilla *et al.*, 1989).

the presence of 1 mM-DTT. Under the last-mentioned assay conditions (2 μ M-rT₃, 1 mM-DTT), but in the presence of lower PTU concentrations (0.1 mM), a 92 and 91 % inhibition of iodothyronine 5'-deiodinating activity was found for liver and brown-adipose-tissue samples respectively. When the inhibitory effect of 1 mM-PTU was studied at high co-substrate (150 mM-DTT) concentration, but lower substrate (rT₃) levels, 92 and 90 % inhibition was found for liver and brown-fat samples at 0.5 μ M-rT₃ respectively and 89 and 91 % inhibition at 2 nM-rT₃ in both the mentioned tissues respectively.

Developmental changes in bovine perirenal-adipose tissue iodothyronine 5'-deiodinase activity

Changes in perirenal-adipose-tissue iodothyronine 5'-deiodinase activity as a function of the pre- and post-natal age are depicted in Fig. 1. Substantial levels of iodothyronine 5'-deiodinase activity are already detectable in the perirenal adipose tissue of bovine fetuses of 59 days gestational age. From around day 150 onwards there is a marked increase in the iodothyronine 5'-deiodinating activity, which reaches maximum levels around days 220–230 and declines suddenly afterwards. This peak value of perirenal-adipose-tissue iodothyro-

nine 5'-deiodinase activity in bovine late-fetal life attain levels that are around 10-fold those of younger or term fetuses. The perirenal-adipose-tissue iodothyronine 5'-deiodinase activity levels are low at birth and from then on they decrease progressively and are hardly detectable 100 days after birth. The iodothyronine 5'-deiodinase activity was quite undetectable in the perirenal adipose tissue of the adult cow.

Iodothyronine 5'-deiodinase activity in different sites of bovine adipose tissue

Results corresponding to the iodothyronine 5'-deiodinase activity in bovine pericardic, peritoneal and intermuscular adipose tissues at different days of life are depicted in Table 2, together with the perirenal data for comparative purposes. As shown in Fig. 1, the pericardic adipose tissue shows substantial levels of 5'-deiodinase activity in the first days of life that are even higher than those found in the perirenal site. The peritoneal and intermuscular sites show lower, but detectable, levels of iodothyronine 5'-deiodinase activity, especially in the second and third days of life. In this same experiment the mesenteric- and retroperitoneal-adipose-tissue sites have yielded very low, but detectable, levels of iodothyronine 5'-deiodinase activity in the first days of life (results not shown). The iodothyronine 5'-deiodinase activity was always undetectable in the subcutaneous adipose tissue studied at the postnatal days of life reported in Table 2. In order to discard the possibility that the enzyme activity found in some adipose-tissue depots was due to contaminating muscle, iodothyronine 5'-deiodinase activity was assayed in muscle samples of newborn calves on different days after birth and no detectable activity was ever found.

DISCUSSION

Results indicated that bovine brown-fat iodothyronine 5'-deiodinase is an enzyme activity with a high K_m for rT₃ (micromolar range) and it is PTU-inhibitable. This pattern of characteristics is not essentially different from that found in bovine liver and led us to conclude that brown-fat 5'-deiodinase in the bovine species, in contrast with that from rodents (Leonard *et al.*, 1983), is essentially a Type I enzyme. This conclusion would be similar to that obtained by Wu *et al.* (1986) in lamb brown-adipose tissue. The persistence of PTU inhibition even in the presence of low co-substrate (1 mM-DTT) or low iodothyronine (2 nM-rT₃) concentrations excludes the possibility of a type II (PTU-resistant, low K_m)

Table 2. Iodothyronine 5'-deiodinase activity in bovine adipose depots different days after birth

Results are means for triplicate assays of samples corresponding to the four indicated adipose depots of individual bovine newborns.

Depot	Time after birth (days)...	Activity (pmol of I ⁻ /h per mg of protein)				
		0	2	3	15	38
Perirenal	73	73	180	384	87	71
Pericardic	296	296	403	345	45	7
Peritoneal	11	11	43	50	—	26
Intermuscular	—	—	35	38	—	7

iodothyronine 5'-deiodinase activity 'mixed' with the type I in bovine adipose tissue. However, the absence of a type II 5'-deiodinase in bovine brown adipose tissue must not be generalized to all the bovine tissues. Pituitary, which possesses a type II enzyme in rodents (Visser *et al.*, 1983), has also been reported to possess a type II 5'-deiodinase activity in the bovine species (Courtin *et al.*, 1985).

The high prenatal iodothyronine 5'-deiodinase activity found here is associated with the reported biochemical characterization of the fetal perirenal depot as brown fat (Casteilla *et al.*, 1987, 1989), whereas the progressive disappearance of the enzyme activity after birth is associated with the postnatal transformation of this adipose depot from a brown- to white-fat appearance in ruminants (Alexander *et al.*, 1975; Casteilla *et al.*, 1987, 1989). The comparative study of the other adipose depots confirms the relationship between the levels of 5'-deiodinase activity found and the presence of characteristic brown fat in different bovine adipose-tissue sites (Casteilla *et al.*, 1987, 1989). The absence of any detectable 5'-deiodinase activity in the subcutaneous site, a depot that presents neither cytologically nor biochemically characteristic features of brown-fat whenever bovine development has been studied (Alexander *et al.*, 1975; Gemmel & Alexander, 1978; Casteilla *et al.*, 1987, 1989) is especially remarkable. Therefore we must conclude that, similarly to what occurs in rodents, the presence of iodothyronine 5'-deiodinase activity in the bovine species is a typical feature of brown adipose tissue when it is compared with white adipose tissue, which lacks significant amounts of this enzyme activity. It must be also noted that, despite the fact that the iodothyronine 5'-deiodinases present in brown fat from the bovine species and rodents are of a different type, data from our laboratory point to a prenatal profile of this enzyme activity in the rat brown fat similar to that reported here for the bovine species (Iglesias *et al.*, 1987; Giralt *et al.*, 1988).

When the possible functional role of the very high iodothyronine 5'-deiodinase activity levels in bovine fetal brown fat is considered, it is worthwhile to compare the present data with our recent study on the sequential expression of the mRNAs corresponding to different components of brown-fat mitochondria in the bovine fetus and newborn (Casteilla *et al.*, 1988). Prenatal iodothyronine 5'-deiodinase activity has an evolution similar to mitochondriogenesis, whereas the maximal 5'-deiodinase activity levels, corresponding to the peak values at the beginning of the third trimester of pregnancy, are attained at the moment when the mRNA coding for the uncoupling protein, the specific component unique to brown-fat mitochondria, first appears (see Fig. 1). The activity of the sympathetic nervous system innervating brown-adipose tissue is known to be the main determinant of the degree of uncoupling-protein-gene expression in adult rodents (Ricquier *et al.*, 1986), whereas only fairly recently has the iodothyronine 5'-deiodinase activity been claimed to be necessary for the cold-induced increase of uncoupling protein mRNA in the adult rat (Bianco & Silva, 1987). However, at the stage of prenatal bovine development when uncoupling-protein mRNA first occurs and iodothyronine 5'-deiodinase have peak values, the sympathetic innervation of brown fat is considered not to be yet developed in ruminants (Gemmel & Alexander, 1978). When all these

facts are taken into account, we are tempted to formulate as a hypothesis for future work that the T_3 generated *in situ*, thanks to the very high 5'-deiodinase activity at the months 6–7 of gestation, may be involved in the prenatal induction of the uncoupling-protein-gene expression in brown fat, whereas the action of the sympathetic activity upon the tissue would act as a *modulator* of this gene expression later in development. In summary, whatever the functional role of the fetal iodothyronine 5'-deiodinase activity is, it is evident that if we consider this enzyme activity as a specific biochemical marker of brown adipose tissue as opposed to white fat, a high iodothyronine 5'-deiodinase activity must be viewed as an event appearing earlier than uncoupling-protein-gene expression in the prenatal differentiation programme of brown fat.

We thank Dr. D. Ricquier for helpful comments and advice. We are grateful to C. Barboiron for taking the samples and C. Tipper for pre-submission editorial assistance. This work was partially supported by Institut National de la Recherche Agronomique, France (Grant 4588) and by Dirección General de Investigación Científica y Técnica, Spain (Grant PB-577/86).

REFERENCES

- Alexander, G., Bennett, J. W. & Gemmell, R. T. (1975) *J. Physiol.* **244**, 223–234
- Bianco, A. C. & Silva, J. E. (1987) *J. Clin. Invest.* **249**, 5421–5427
- Casteilla, L., Forest, C., Ricquier, D., Robelin, J., Lombet, A. & Ailhaud, G. (1987) *Am. J. Physiol.* **252**, E627–E636
- Casteilla, L., Champigny, O., Bouillaud, F., Robelin, J. & Ricquier, D. (1989) *Biochem. J.* **257**, 665–671
- Courtin, F., Pelletier, G. & Walker, P. (1985) *Endocrinology (Baltimore)* **117**, 2527–2533
- Fernández, J. A., Mampel, T., Villarroja, F. & Iglesias, R. (1987) *Biochem. J.* **243**, 281–284
- Gemmel, R. T. & Alexander, G. (1978) *Aust. J. Biol. Sci.* **31**, 505–515
- Giralt, M., Villarroja, F., Mampel, T. & Iglesias, R. (1986) *Biochem. Biophys. Res. Commun.* **138**, 1315–1321
- Giralt, M., Martin, I., Mampel, T., Villarroja, F., Iglesias, R. & Viñas, O. (1988) *Biochem. Biophys. Res. Commun.* **156**, 493–499
- Goswami, A. & Rosenberg, I. N. (1986) *Endocrinology (Baltimore)* **119**, 916–923
- Iglesias, R., Fernandez, J. A., Mampel, T., Obregón, M. J. & Villarroja, F. (1987) *Biochim. Biophys. Acta* **923**, 233–240
- Kates, A. & Himms-Hagen, J. (1985) *Biochem. Biophys. Res. Commun.* **130**, 188–193
- Kochupillai, N. & Yallow, R. S. (1978) *Endocrinology (Baltimore)* **102**, 128–135
- Larsen, P. R., Silva, J. E. & Kaplan, M. M. (1981) *Endocr. Rev.* **2**, 87–102
- Lean, M. E. J. & James, W. P. T. (1986) in *Brown Adipose Tissue* (P. Trayhurn & D. Nicholls, eds.), pp 339–366, Edward Arnold, London
- Leonard, J. L. & Rosenberg, I. N. (1980) *Endocrinology (Baltimore)* **107**, 1376–1383
- Leonard, J. L., Mellen, S. A. & Silva, J. E. (1983) *Endocrinology (Baltimore)* **112**, 1153–1155
- Nicholls, D., Cunningham, S. A. & Rial, E. (1986) in *Brown Adipose Tissue* (P. Trayhurn & D. Nicholls, eds.), pp 52–85, Edward Arnold, London

- Ricquier, D., Bouillaud, F., Toumelin, P., Mory, G., Bazin, R., Arch, J. & Penicaud, L. (1986) *J. Biol. Chem.* **30**, 13905–13910
- Silva, J. E. & Larsen, P. R. (1983) *Nature (London)* **305**, 712–713
- Strieleman, P. J., Gribskov, C. L., Kemnitz, J. W., Schalinske, K. L., Claude, P., Parada, I., Shrago, E. & Swick, R. W. (1985) *Comp. Biochem. Physiol.* **81B**, 393–399
- Visser, T. J. (1979) *Biochim. Biophys. Acta* **569**, 302–308
- Visser, T. J., Leonard, J. L., Kaplan, M. M. & Larsen, P. R. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5080–5084
- Visser, T. J., Kaplan, M. M. & Leonard, T. J. (1983) *J. Clin. Invest.* **71**, 992–1002
- Wu, S. Y., Polk, D. H. & Fisher, D. A. (1986) *Endocrinology (Baltimore)* **118**, 1334–1339
- Zak, B. & Cohen, J. (1961) *Clin. Chim. Acta* **6**, 665–676

Received 28 July 1988/2 November 1988; accepted 10 November 1988