Tissue specificity of rat mitochondrial dimethylglycine dehydrogenase expression

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Expression of mitochondrial dimethylglycine dehydrogenase (Me_2GlyDH) was analysed in various tissues, liver cell types and developmental stages of the rat. Total RNA extracted from liver, spleen, brain, kidney, lung and heart was reverse-transcribed into cDNA and amplified with Me_2GlyDH cDNA-specific oligonucleotides by PCR. Expression of the enzyme was observed mainly in liver and kidney. In addition, Me_2GlyDH mRNA could be demonstrated in total RNA samples of lung, heart and brain but was barely detectable in spleen total RNA. In RNA prepared from 14-day rat embryos, Me_2GlyDH-specific mRNA was clearly present. Among various liver cell types, besides hepatocytes, endothelial cells showed a high level of Me_2GlyDH mRNA expression. There was no amplification product detectable in liver macrophages (Kupffer cells) and only a very faint one in fat-storing cells (Ito cells). Western blots confirmed at the protein level the predominant expression of the enzyme in liver and kidney, but Me_2GlyDH protein was also present in the protein extract of lung, heart, spleen and brain. Immunohistochemical staining of liver slices with Me_2GlyDH-specific antiserum revealed that expression of this enzyme is evenly distributed throughout the liver tissue. In the kidney, expression of the enzyme was located in the proximal tubule cells. Our results demonstrate that, contrary to the previously assumed liver-restricted expression, this enzyme is specifically expressed predominantly in the liver and kidney, but, in addition, it is detectable in many other tissues of the rat.

INTRODUCTION

Choline, an essential dietary component for mammals, may have two metabolic fates. It can be phosphorylated to phosphocholine and committed to the biosynthetic pathway leading to phosphatidylcholine. Alternatively, it can enter the mitochondria and be oxidatively degraded to glycine (Scheme 1). At physiological concentrations, choline enters the mitochondria via a specific transporter [1] which has been shown to be the major site for control of choline oxidation in isolated rat liver mitochondria. The first enzyme of the choline oxidation pathway, choline dehydrogenase, an oligomeric protein complex of the inner mitochondrial membrane, has been shown in both rats and humans to be expressed mainly in liver and kidney [2]. Choline dehydrogenase is a flavoenzyme which contains FAD as prosthetic group; betaine aldehyde formed in the choline dehydrogenase reaction is converted into betaine by the NAD^+-linked betaine aldehyde dehydrogenase, an enzyme of the mitochondrial matrix [3]. In the kidney, betaine plays an important role as an organic osmolyte [4]. In the mammalian liver, betaine is an essential methyl-group donor for the synthesis of methionine from homocysteine, a reaction catalysed by betaine–homocysteine methyltransferase. This enzyme was recently shown to be located in the cytoplasm of liver and kidney cells of man and pig, but only in the liver of the rat [5]. Betaine thus has to efflux from the mitochondria into the cytoplasm of the liver cell in order to function as a methyl donor in the synthesis of methionine. Porter et al. [6] showed recently that efflux of betaine from the mitochondrion is a simple diffusion process which is proportional to the intramitochondrial concentration of the substance. Betaine is transformed into dimethylglycine in the betaine–homocysteine methyltransferase reaction. The last two reactions in the oxidative pathway from choline to glycine, catalysed by dimethylglycine dehydrogenase (Me_2GlyDH) and sarcosine dehydrogenase, are located in the mitochondrial matrix [7,8]. Thus dimethylglycine must be transported back across the inner mitochondrial membrane into the matrix. This may be a diffusion-dependent process, as it has been shown that the diffusion rates of betaine, dimethylglycine, sarcosine and glycine across the mitochondrial membrane increase with decreasing degree of *N*-methylation of these compounds [6]. Me_2GlyDH and sarcosine dehydrogenase have been identified as the folate-binding proteins of the hepatocyte mitochondrial matrix and were thought to be present in rats exclusively in the liver [7–9].

We have previously described the characterization of a Me_2GlyDH cDNA clone [10]. With the aid of oligonucleotides derived from this cDNA and Me_2GlyDH-specific antiserum, we investigated the expression of this enzyme at the mRNA and protein level in various tissues, liver cells and developmental stages of the rat. Contrary to previous reports, our results demonstrate that Me_2GlyDH is expressed at increased levels in both liver and kidney, and that detectable levels of expression can be demonstrated at the RNA and protein level in heart, brain, lung and spleen also. Within the liver, besides high levels of expression in hepatocytes, endothelial cells also expressed the enzyme, but liver macrophages (Kupffer cells) and fat-storing cells (Ito cells) showed no or a very low level of expression respectively. Immunohistochemical analysis of liver slices revealed an even expression of the enzyme throughout the hepatocyte population. In the kidney, expression of Me_2GlyDH was located to the cells of the proximal tubule.

EXPERIMENTAL

Plasmids and *in vitro* transcription of RNA

The Me_2GlyDH cDNA corresponding to the coding sequence of the mature enzyme was cloned as an *Eco*RI–*Hin*dII fragment into the corresponding sites of plasmid pSPT19 (Boehringer, Mannheim, Germany) giving rise to plasmid pSPT19xcDDH1. The second plasmid construct employed in this work contained

Abbreviations used: Me_2GlyDH, dimethylglycine dehydrogenase (EC 1.5.99.2); ABC, avidin–biotin complex.
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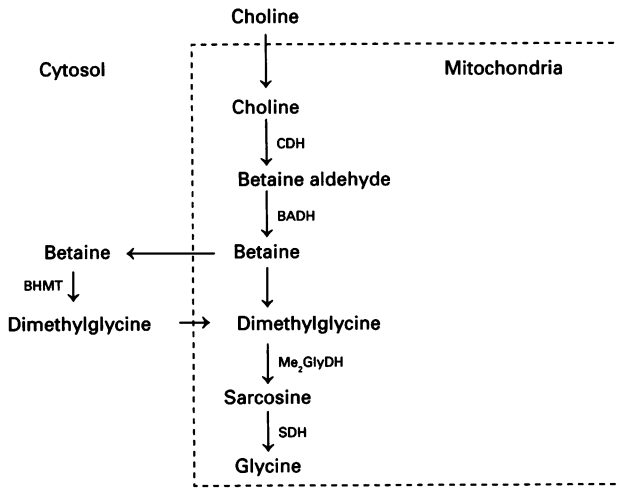

Scheme 1 Pathway for the oxidative degradation of choline to glycine

CDH, choline dehydrogenase; BADH, betaine aldehyde dehydrogenase; BHMT, betaine–homocysteine methyltransferase; Me_2GlyDH, dimethylglycine dehydrogenase; SDH, sarcosine dehydrogenase. For further details, see the text.

a *Sma*I deletion introduced into the coding sequence of the Me_2GlyDH cDNA. This internal deletion removes 260 nucleotides between nucleotide 289 and 549 of the cDNA. The resulting plasmid construct was designated pSTPxcDDH1delta*Sma*I. RNA encoding the Me_2GlyDH protein was transcribed from these plasmids by SP6 polymerase after linearization of the DNA with *Hin*dIII. The two plasmid DNAs and the full-length and truncated transcripts were employed as internal standards in PCR.

Plasmid DNA was propagated in and isolated from *Escherichia coli* JM109 [11].

Source of biological tissue and cells

Liver, spleen, lung, heart, kidney and brain tissue samples were taken from 3-month-old female Wistar rats. For monitoring Me_2GlyDH expression during ontogeny, rat embryos were taken 14 days *post coitum*. In addition, livers and kidneys were taken from 7-day- and 18-day-old rats.

The cell-type-specific expression of Me_2GlyDH within the liver was performed on primary cultures of hepatocytes, fat-storing cells (Ito cells), liver macrophages (Kupffer cells) and sinusoidal endothelial cells. Primary cultures of hepatocytes were established as described in [12]; Ito cells were prepared and cultured as described in [13]; Kupffer cells and sinusoidal endothelial cells were prepared as described by Brouwer et al. [14] as modified by Eyhorn et al. [15]. After 48 h in culture, the cells were counted, rinsed three times with PBS and frozen in liquid nitrogen. They were then used for total RNA extraction.

RNA extraction, Northern-blot hybridization and DNA PCR

Total RNA was extracted from rat tissue and cell cultures as described in [16], including a DNAase step. The RNA preparations were analysed on 1% formaldehyde–agarose gels, and the intactness of the RNA was judged by the presence in equal amounts of the 18 S and 28 S ribosomal RNA bands on the ethidium bromide-stained gel (results not shown). Northern blots with rat liver RNA and hybridization to [^{32}P]dCTP random primer-labelled rat β-actin cDNA and rat Me_2GlyDH cDNA-derived probes were performed by standard protocols [11].

First-strand cDNA synthesis from total RNA was achieved with a commercial kit purchased from Pharmacia (Freiburg, Germany). Amplification of the synthesized cDNA was performed with *Taq* DNA polymerase (Amersham, Braunschweig, Germany) and the following oligonucleotides: a 5′ and 3′ β-actin cDNA-specific oligonucleotide (5′-TCTACAATGAGCTGCGTGTGG-3′ and 5′-CCATAGGCAGAATGTCAGGA-3′ respectively) and a 5′ and 3′ Me_2GlyDH cDNA-specific oligonucleotide (5′-GAAGGACAGGAAAGCCCACC-3′ and 5′-CCATAGGCAGAATGTCAGGA-3′ respectively). The reaction conditions for the PCR were as described by the supplier of the first-strand cDNA-synthesis kit (Pharmacia). As internal standard either pSPTxcDDH1delta*Sma*I or RNA transcribed from this plasmid *in vitro* was added to the samples as described in the legends to the Figures. *Taq* DNA polymerase was added to the incubation mixtures preheated to 93 °C. Annealing of the oligonucleotides was at 63 °C for 15 s, followed by DNA synthesis at 72 °C for 2 min and melting of the hybrids at 93 °C. The PCR cycle was repeated 35 times. The PCR products were analysed by gel electrophoresis on 1% agarose gels, stained with ethidium bromide, visualized under u.v. light and photographed using a Polaroid camera.

Immunoprecipitation of Me_2GlyDH and Western blots

For the analysis of Me_2GlyDH expression at the protein level, liver, kidney, spleen, brain, heart and lung were taken from 3-month-old female Wistar rats. The tissues were immediately frozen in liquid nitrogen, crushed to a powder and homogenized in a glass Potter–Elvehjem homogenizer in 50 mM Tris/HCl, pH 7.4, buffer containing 100 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol and 1 mM phenylmethanesulphonyl fluoride. The homogenate was centrifuged at 12000 ***g***, and 70 μg of the protein from the supernatant was analysed for the presence of Me_2GlyDH by either immunoprecipitation or Western blot. For immunoprecipitations 70 μg of supernatant protein was diluted into 5 ml of the homogenization buffer without 2-mercaptoethanol. Then 10 μl of Me_2GlyDH-specific antiserum was added, and the sample incubated at 4 °C overnight with gentle agitation. Then 5 mg of Protein A–Sepharose (Pharmacia) was added and incubation continued at room temperature for 2 h. The Protein A–Sepharose was collected by centrifugation at 10000 ***g***, washed three times with the same buffer and the Protein A–Sepharose-bound protein was eluted by boiling in 100 μl of SDS/PAGE sample buffer. The Sepharose was pelleted by centrifugation and the supernatant applied to a 7.5% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue. When Western blots were employed, 70 μg of the supernatant from the tissue homogenates was separated on a 7.5% polyacrylamide gel. After electrophoresis, the separated proteins were blotted on to nitrocellulose paper, and the Western blot developed with Me_2GlyDH-specific antiserum and alkaline phosphatase-coupled second antibody as described by the supplier (Bio-Rad, Munich, Germany).

Immunohistochemical methods

Wistar rats (150 g, female) were perfused through the portal vein under ether anaesthesia with periodate–lysine/paraformaldehyde solution at pH 7.4. Cryostat sections of liver and kidney (6 μm thick) were cut, dried in air, fixed in acetone for 10 s and subjected to the immunohistochemical procedure. The tissue sections were incubated with Me_2GlyDH-specific antiserum

(1:20), washed in PBS and treated with the avidin–biotin complex (ABC) peroxidase kit (Camon, Wiesbaden, Germany) according to the instructions of the supplier. All specimens were finally mounted in gelatine.

RESULTS

Northern-blot analysis of total rat liver RNA and immunoprecipitation of Me_2GlyDH from rat liver extract

Northern blots of rat total liver RNA hybridized to a ^{32}P-labelled Me_2GlyDH cDNA probe gave no hybridization signal (Figure 1a, lane 2). When the same Northern blot was hybridized with an actin cDNA-specific probe, the expected signal was readily detectable (Figure 1a, lane 1). Immunoprecipitation of liver cell extract with Me_2GlyDH-specific antiserum revealed on Coomassie Brilliant Blue-stained polyacrylamide gels the presence of the Me_2GlyDH protein band (Figure 1b). From the intensity of the stained Me_2GlyDH on the polyacrylamide gel, we assume that the enzyme is not a protein of low abundance in the liver. Therefore it seems that transcription of the Me_2GlyDH gene is low or the transcribed mRNA instable.

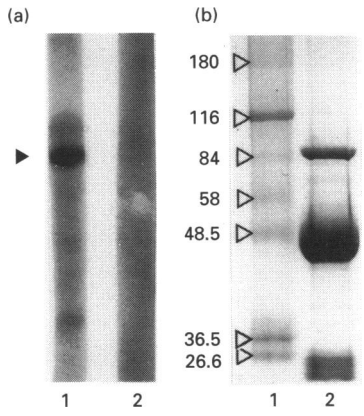

Figure 1 Northern-blot of rat liver RNA and immunoprecipitation of rat liver Me_2GlyDH

Hybridization of actin-specific and Me_2GlyDH-specific probes to Northern blots of total rat liver RNA and immunoprecipitation of rat liver extract with Me_2GlyDH-specific antiserum was performed as described in the Experimental section. (**a**) Total liver RNA hybridized to the actin-specific probe (lane 1) and to the Me_2GlyDH-specific probe (lane 2). (**b**) Lane 1, molecular-mass markers (in kDa); lane 2, immunoprecipitate of liver Me_2GlyDH.

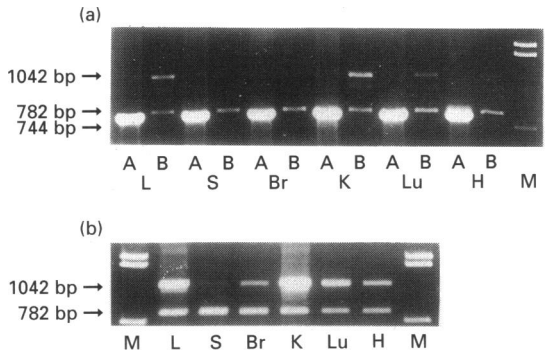

Figure 2 Levels of Me_2GlyDH mRNA expression in various rat tissues as demonstrated by reverse transcription PCR

(**a**) Total RNA (1 μg sample) extracted from various rat tissues was mixed with 1 pg of pSPTxcDDH1delta*Sma*I DNA as internal control and reverse-transcribed into cDNA with the aid of a commercially available cDNA-synthesis kit (Pharmacia). The cDNA samples were then amplified in a competitive PCR with the actin- (A) and Me_2GlyDH- (B) specific primers (see the Experimental section). 1042 bp = Me_2GlyDH DNA fragment amplified from total RNA of rat tissues; 782 bp = *Sma*I-truncated DNA fragment amplified from the pSPTxcDDHdelta*Sma*I DNA; 744 bp = amplified actin DNA fragment. L, liver; S, spleen; Br, brain; K, kidney; Lu, lung; H, heart; M, DNA size markers. (**b**) Total RNA (1 μg sample) extracted from various tissues was mixed with 25 pg of *in vitro* SP6-polymerase-transcribed Me_2GlyDH1delta*Sma*I mRNA generated from *Hin*dIII-linearized pSPT19xcDDH1delta*Sma*I, reverse-transcribed into cDNA and amplified with the Me_2GlyDH-specific primers. This RNA standard allowed the reverse transcription to be monitored also.

Amplification of Me_2GlyDH-specific RNA from various rat tissues by reverse transcription and PCR

Because of the low level of Me_2GlyDH mRNA, we analysed expression of the Me_2GlyDH gene in different rat tissues using the PCR technique. Total rat RNA from liver, spleen, brain, kidney, lung and heart was first reverse-transcribed into cDNA and then amplified with the oligonucleotide pairs described in the Experimental section. The primers selected from the Me_2GlyDH cDNA sequence gave, on amplification, a 1042 bp DNA fragment. As a positive control, two oligonucleotide primers derived from the ubiquitously expressed β-actin gene were employed. Amplification of the β-actin cDNA with this primer pair resulted in a 744 bp DNA fragment. Control reactions with RNA samples not transcribed into cDNA gave no amplification product. For a semiquantitative evaluation of the reaction products, 1 pg of pSPT19xcDDH1delta*Sma*I DNA was added to the reverse-transcribed samples before amplification with the Me_2GlyDH-specific primers. From this internally deleted cDNA construct, a 782 bp DNA product was amplified with the Me_2GlyDH primers. As a control for the reverse transcription reaction, 25 pg of *in vitro* transcribed pSPTxcDDH1delta*Sma*I RNA was added to the RNA samples before reverse transcription in alternative series of reverse transcription-PCR.

Figure 2 illustrates the tissue-specific distribution of Me_2GlyDH expression in the rat. The main tissues that expressed the enzyme were liver and kidney. The DNA fragment amplified from the reverse-transcribed RNA with the Me_2GlyDH-specific primer pair migrated identically with the DNA fragment amplified with the same oligonucleotides from authentic Me_2GlyDH cDNA and exhibited the same restriction enzyme pattern (not shown). In addition to liver and kidney, detectable levels of Me_2GlyDH expression were present in all the organs analysed except spleen (Figure 2a). A comparison of the expression of the enzyme in different tissues according to the internal RNA standard showed that, after kidney and liver, lung exhibited the highest level of expression, followed by heart and brain (Figure 2b). The specificity of Me_2GlyDH expression within various liver cell types is presented in Figure 3. As expected hepatocytes showed the highest level of expression (Figure 3a). Quantification according to the internal RNA standard revealed that endothelial cells also expressed the enzyme mRNA to an appreciably high level (Figure 3b). No Me_2GlyDH cDNA-specific amplification product was detectable in RNA from liver macrophages (Kupffer cells) and only a very faint amplification signal in RNA from fat-storing cells (Ito cells).

The basic importance of this enzyme of choline catabolism in rat was demonstrated by the fact that its mRNA was readily detectable in total RNA samples extracted from 14-day whole embryos (Figure 4). As a control the amplification product obtained from a sample prepared from total liver RNA of 18-day-old rats is shown (Figure 4a, lane 2). A comparison of Me_2GlyDH expression in liver and kidney from 1-week-old rats

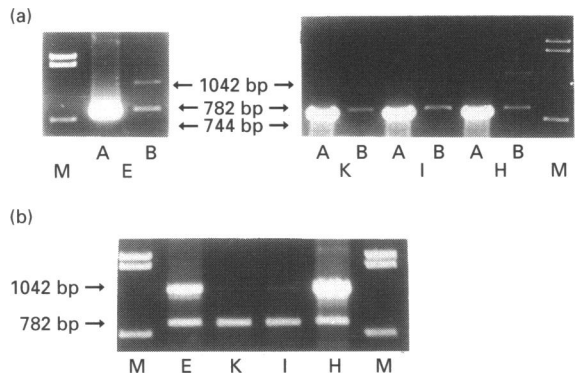

Figure 3 Levels of Me_2GlyDH mRNA expression in individual liver cell types

(**a**) A 1 μg sample of total RNA extracted from various liver cell types was mixed with 1 pg of pSPTxcDDH1delta*Sma*I DNA as internal standard, reverse-transcribed and competitively amplified as described in the legend to Figure 1. Lanes A, amplification products obtained with actin primers; lanes B, amplification products obtained with the Me_2GlyDH primers. (**b**) Total RNA (1 μg sample) extracted from various liver cell types was mixed with 25 pg of Me_2GlyDH1delta*Sma*I RNA and co-reverse-transcribed and co-amplified as described in the legend to Figure 1. E, Endothelial cells; K, Kupffer cells; I, Ito cells; H, hepatocytes; M, DNA molecular mass markers.

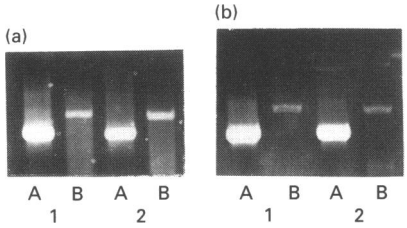

Figure 4 Expression of Me_2GlyDH mRNA during the ontogeny of the rat

Total RNA (0.5 μg) was reverse-transcribed into cDNA and amplified with the aid of rat β-actin cDNA-specific (A) and rat Me_2GlyDH cDNA-specific (B) oligonucleotides as described in the legend to Figure 1. (**a**) Lane 1, amplification products obtained from 0.5 μg of total RNA isolated from embryos 14 days *post coitum*; lane 2, amplification products obtained from 0.5 μg of total RNA isolated from the livers of 18-day-old rats. (**b**) Me_2GlyDH expression in liver (lane 1) and kidney (lane 2) of 7-day-old rats.

Figure 5 Analysis of Me_2GlyDH expression at the protein level

Protein (70 μg) extracted from liver (lane 1), spleen (lane 2), brain (lane 3), kidney (lane 4), lung (lane 5) and heart (lane 6) were separated by PAGE on a 7.5% gel, blotted on to nitrocellulose paper and the Western blot was developed with Me_2GlyDH-specific antiserum and alkaline phosphatase-coupled second antibody as described by the supplier (Bio-Rad).

demonstrated that, at this stage after birth, there was no difference among the tissues in the levels of expression of the enzyme mRNA (Figure 4b).

Protein levels of Me_2GlyDH in various rat tissues

In order to correlate expression at the RNA level with enzyme protein levels, we analysed rat tissue extracts for the presence of

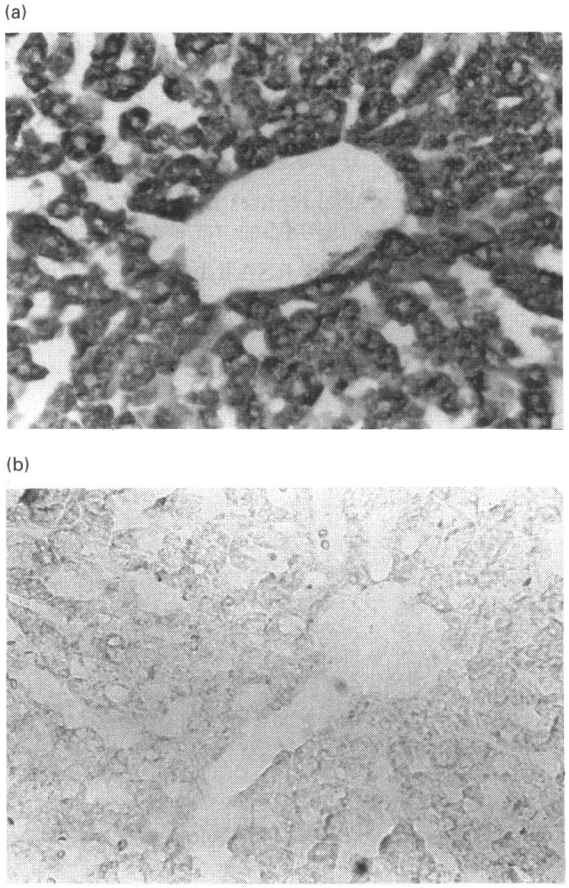

Figure 6 Distribution of Me_2GlyDH within rat liver

(**a**) Immunohistochemical demonstration of Me_2GlyDH in the liver parenchyma of a Wistar rat. The hepatocytes show a marked positivity with a dark reaction product in their cytoplasm. The ABC technique was used. Primary magnification: × 125. (**b**) Negative control with preimmune serum for the immunohistochemical reaction from the liver of a Wistar rat. The ABC technique was used. Primary magnification: × 125.

Me_2GlyDH by Western blots. This analysis confirmed the RNA data and showed that highest levels of expression of Me_2GlyDH were observed in the liver and kidney (Figure 5). However, as observed by PCR performed with cDNA reverse-transcribed from tissue RNA samples, there were detectable levels of the protein in other tissues as well. In spleen and brain, the correlation between RNA and protein levels was poor. There is a clear protein band detectable in spleen extracts, although the signal at the RNA level is only faint. The reverse applies to brain. The spleen is known for its high concentration of RNAases, and the high lipid content of the nervous tissue may represent a difficulty in extraction of Me_2GlyDH.

Immunohistochemical analysis of Me_2GlyDH expression within the liver and kidney

Expression of many enzymes has been shown to be zonally distributed in the liver [17]. Immunohistochemical staining of liver slices with Me_2GlyDH-specific antiserum revealed no such distribution for Me_2GlyDH. Expression was observed in hepatocytes throughout the liver parenchyma (Figure 6). A different picture emerged during immunohistochemical analysis of kidney slices. Me_2GlyDH was predominantly present in the proximal tubules (Figure 7).

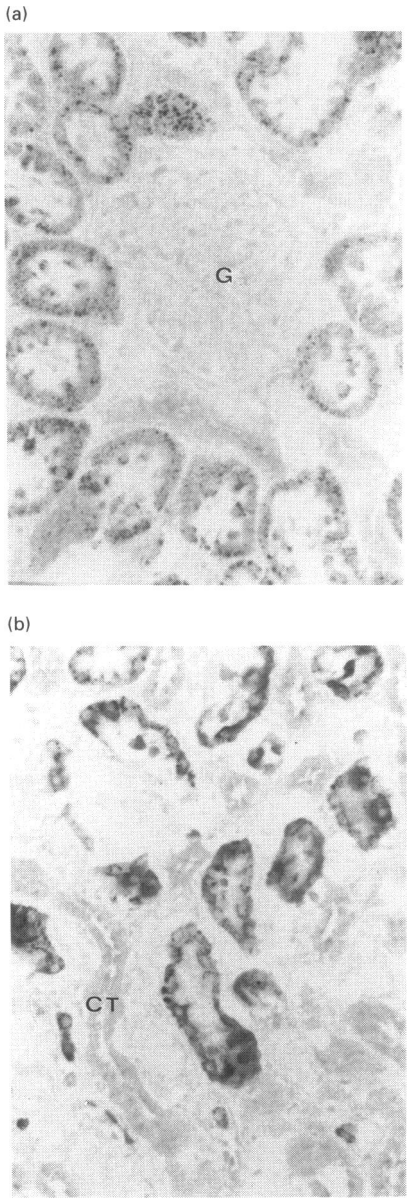

Figure 7 Distribution of Me_2GlyDH within rat kidney

(**a**) Immunohistochemical detection of Me_2GlyDH (dark reaction product) in the epithelia of proximal tubules of the renal cortex (Wistar rat). Note the completely negative finding in the glomerulum (G). The ABC technique was used. Primary magnification: ×125. (**b**) Immunohistochemical demonstration of Me_2GlyDH in cells of the distal tubules of the renal cortex (dark reaction product) of a Wistar rat in the border region to the medulla. Note the negative immunoreactivity of the collecting tubules (CT). The ABC technique was used. Primary magnification: ×125.

DISCUSSION

Me_2GlyDH and the related enzyme sarcosine dehydrogenase [8] catalyse the two last steps in the degradation of choline to glycine. These two enzyme reactions are located in the mitochondrial matrix, and in both enzymes the cofactor FAD is covalently linked to a histidine residue of the polypeptide chain. We are interested in tissue-specific factors regulating translation and maturation of the mitochondrial precursor form of these enzymes. With the aid of the previously isolated cDNA for Me_2GlyDH [10], we undertook a systematic analysis of the tissue- and cell-type-specific expression of this enzyme. From immunoprecipitation experiments and $(NH_4)_2SO_4$ precipitation of liver mitochondrial extracts as well as from the published purification procedures of the enzyme from rat liver [8], one can assume that Me_2GlyDH is a relatively abundant protein in this organ. As Northern blots of total liver RNA hybridized with a Me_2GlyDH cDNA-specific probe gave no hybridization signal, we assume that either transcription of the gene takes place at a low level or that the mRNA is unstable. We cannot exclude the possibility that transcription of the gene takes place only under certain conditions but that the translated and translocated protein is stable in the liver mitochondrion.

When total RNA was transcribed into cDNA, we could clearly show by the PCR technique that the enzyme is expressed in both liver and kidney at approximately the same level. Several transcription factors, such as hepatic nuclear factor (HNF)-1 or HNF-4, are active in liver and kidney [18], which corresponds to the tissue-specific expression of choline dehydrogenase, betaine aldehyde dehydrogenase and betaine–homocysteine methyltransferase as described for man and pig [5]. Our results indicate that, in contrast with previous reports [7–9], Me_2GlyDH is expressed in the kidney in accordance with the tissue distribution of the other enzymes of choline catabolism.

Analysis of individual liver cell types for expression of Me_2GlyDH at the cDNA level indicated that epithelial cells have this ability. Thus expression of this enzyme is not only liver- and kidney-specific, but also characteristic of liver endothelial cells. It was not possible to analyse the expression of Me_2GlyDH in epithelial cells of other origin. It cannot, however, be excluded that the low level expression found in lung, heart, brain and spleen is due to the expression of Me_2GlyDH in the endothelial components of these tissues.

In the liver, expression of enzyme is evenly distributed among hepatocytes. In the kidney, however, there is a correlation between the distribution of the expression of Me_2GlyDH and the distribution of betaine. Betaine is one of several organic osmolytes and is synthesized from choline by proximal tubule and renal medullary cells. After salt loading, rats accumulate large amounts of betaine in their renal medullas (for a review see [4]). The correlation in the distribution of betaine and Me_2GlyDH within the kidney may indicate that at least some of the betaine accumulated in the kidney can be further oxidized by this tissue to glycine via Me_2GlyDH and sarcosine dehydrogenase.

Me_2GlyDH was found to be expressed in 14-day rat embryos. This underlines the basic importance of this enzyme and the choline-catabolic pathway in general. It may be assumed that the other enzymes of the pathway are also expressed early during embryogenesis. Our results show that Me_2GlyDH exhibits an interesting pattern of developmentally and tissue-specific regulated expression.

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