Identification of the two histidine residues responsible for the inhibition by malonyl-CoA in peroxisomal carnitine octanoyltransferase from rat liver

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Abstract Carnitine octanoyltransferase (COT), an enzyme that facilitates the transport of medium chain fatty acids through peroxisomal membranes, is inhibited by malonyl-CoA. cDNAs encoding full-length wild-type COT and one double mutant variant from rat peroxisomal COT were expressed in *Saccharomyces cerevisiae*. Both expressed forms were expressed similarly in quantitative terms and exhibited full enzyme activity. The wild-type-expressed COT was inhibited by malonyl-CoA like the liver enzyme. The activity of the enzyme encoded by the double mutant H131A/H340A was completely insensitive to malonyl-CoA in the range assayed (2–200 μM). These results indicate that the two histidine residues, H131 and H340, are the sites responsible for inhibition by malonyl-CoA. Another mutant variant, H327A, abolishes the enzyme activity, from which it is concluded that it plays an important role in catalysis.

Key words: Malonyl-coenzyme A; Peroxisome; Carnitine octanoyltransferase; Inhibitory kinetics; Site-directed mutagenesis; Rat liver

1. Introduction

Carnitine octanoyltransferase (COT) is a member of the family of carnitine acyltransferases that transfer fatty acyl groups from carnitine to CoA and vice versa and thus facilitate the transport of fatty acids through various organelles for further metabolization. COT is present in subcellular organelles, like the endoplasmic reticulum and peroxisomes [1–3]. This feature may be derived from the capacity of the gene to produce two proteins through a process of *trans*-splicing [4]. In peroxisomes, COT activity is inhibitable by malonyl-CoA [5] in a way similar to the mitochondrial carnitine palmitoyltransferase (CPT) I. In contrast, mitochondrial CPT II is not inhibitable by malonyl-CoA.

Inhibition of carnitine acyltransferase activities by malonyl-CoA is produced by two sites of malonyl-CoA interaction: one involves the catalytic acyl-CoA binding domain, and the other, which has a greater capacity for binding and regulation by malonyl-CoA, behaves as an allosteric component [6–9]. The involvement of His residues in the inhibitory modulation by malonyl-CoA has been suggested [10,11]. Recently, several attempts have been made to identify the domains and the histidine or histidines of CPT I that bind malonyl-CoA but they remain to be clarified [12,13].

In this study, a double mutant version and the wild-type of the COT enzyme have been expressed in the budding yeast *Saccharomyces cerevisiae* and several parameters, including malonyl-CoA sensitivity, have been analyzed. This approach led us to identify H131 and H340 as the residues involved in the response to malonyl-CoA, since their mutation abolishes malonyl-CoA inhibition.

2. Materials and methods

2.1. Expression of COT cDNA in S. cerevisiae

The rat liver clone λ2AxCOT, provided by Dr. Chatterjee [14], was used as a probe in the screening of a λgt10 rat liver cDNA library 5′-stretch from adult male Sprague–Dawley rats (Clontech). Clone λgtCOT14 was subcloned into the *Eco*RI site of plasmid pBluescript (pBS-COT) and sequenced. This fragment, which encompasses the entire coding sequence for COT and includes nt 33–2670 of the previously reported sequence U26033 [15], was used for the expression experiments. The coding region of COT was subcloned into the *S. cerevisiae* expression plasmid pYES2 (Invitrogen) as follows. The NH_2-terminal end of the open reading frame (nt 52–509) was amplified by PCR using plasmid pBS-COT. The forward oligonucleotide 5′-TCG ATA AGC TTA TAA AAT GGA AAA TCA ATT GGC TAA G-3′ includes an extra *Hin*dIII restriction site (underlined) for subcloning into the expression vector and a consensus sequence (bold), optimized for efficient translation, upstream of the *S. cerevisiae* start codon. Oligonucleotide 5′-GGT TCA TGT CTA GAG CAG-3′ was used for reverse priming (*Xba*I site underlined). The amplification fragment was digested with *Hin*dIII-*Xba*I, cloned in pYES2 and sequenced to confirm the absence of mutations. The construct was digested with *Xba*I and ligated with the fragment *Xba*I-*Xba*I (nt 510–2009) to yield pYESCOTwt.

For expression in yeast cells, the wild-type strain YPH499 (*MATa ura3-52 leu2-D1 ade2-101 lys2-801 his3-D200 trp1-D63*) was transformed with different constructs [16], and maintained in complete minimal medium lacking uracil (CM(−ura)) with 2% glucose as carbon source [17]. For COT expression, cells from late exponential cultures were recovered and grown for 12 h in CM(−ura) with galactose as carbon source. Cells were then recovered by centrifugation at 2000×*g* for 5 min at 4°C, washed in 20 ml of ice-cold buffer A (150 mM KCl, 5 mM Tris–HCl pH 7.2), centrifuged again, and resuspended in 1 ml of buffer B (buffer B is buffer A supplemented with 1 mM PMSF, 0.5 mM benzamidine, 10 ng/ml leupeptin, 100 ng/ml pepstatin). 0.5 ml of cold, acid-washed glass beads was added to each

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Abbreviations: COT, carnitine octanoyltransferase; CPT, carnitine palmitoyltransferase; CM(−ura), complete minimal medium lacking uracil

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sample and cells were disrupted by vigorous vortexing (four pulses of 1 min, samples were kept on ice for 1 min after each pulse). Homogenates were centrifuged at about 800×*g* for 3 min at 4°C, to remove glass beads and cell debris. This crude extract was further centrifuged at 15 000×*g* for 10 min at 4°C. Pellets and supernatants were immediately frozen and stored at −20°C.

2.2. Site-directed mutagenesis

Site-directed mutagenesis of histidines 131, 327 and 340 to alanine was carried out by the megaprimer method using asymmetric PCR [18] and plasmid pYESCOTwt. The following oligonucleotides were used: H131A: 5′-GCA TAC TAC TGT GGG CCA ACT TGA ACT ACT GGC-3′; H327A: 5′-GGC TGT AGC TGT GAT GCT GCT CCT TAT GAT GC-3′ and H340A: 5′-GGA GCT TCT CAT CAA CGT ACG CAG CAA TGT TCA CCA TAA-3′. The mutated nucleotides are underlined. To obtain the megaprimer fragments, the oligonucleotides were COT57*for*: 5′-TCG ATA AGC TTA TAA ACA ATT GGC TAA G-3′; COT520*rev*: 5′-TCA TGT CTA GAG TAT TTC CAG ATT TAT G-3′; COT734*for*: 5′-GCT GGA AAT GAA CCT GTT GGG-3′ and COT1221*rev*: 5′-GCT GCT TTG AGG TGT TGG GC-3′. The mutated fragments were used to construct the plasmids pYESCOT^{H327A} and the double mutant pYESCOT$^{H131A/H340A}$. The appropriate substitutions, as well as the absence of unwanted mutations, were confirmed by sequencing the inserts with an Applied Biosystems 373 automated DNA sequencer.

2.3. Determination of carnitine acyltransferase activity

Carnitine acyltransferase activity was determined by the radiometric method as described in [19] with minor modifications. The substrates were decanoyl-CoA and L-[methyl-^{3}H]carnitine. Enzyme activity was assayed for 4 min at 30°C in a total volume of 200 μl. The protein sample, 40 μl (∼2 μg), was pre-incubated for 1 min, and then 160 μl of the reaction mixture was added. The final concentrations were 105 mM Tris–HCl (pH 7.2), 2 mM KCN, 15 mM KCl, 4 mM $MgCl_2$, 4 mM ATP, 250 μM reduced glutathione, 50 μM decanoyl-CoA, 400 μM L-[methyl-^{3}H]carnitine (0.3 μCi) and 0.1% defatted bovine albumin. Reactions were stopped by the addition of 200 μl of HCl 1.2 N, and the product acyl-L-[methyl-^{3}H]carnitine was extracted with water-saturated *n*-butanol. K_m values were estimated by analyzing the data from three experiments performed in triplicate using the program Enzfit (Biosoft). When malonyl-CoA inhibition was assayed, increasing concentrations of malonyl-CoA were included. All protein concentrations were determined using the Bio-Rad protein assay with bovine albumin as standard.

Liver rat peroxisomes were isolated in a Nycodenz cushion as previously described [20]. The peroxisomes were dispersed in 250 mM sucrose, 10 mM Tris–HCl pH 7.4 and 1 mM EDTA and stored at −20°C.

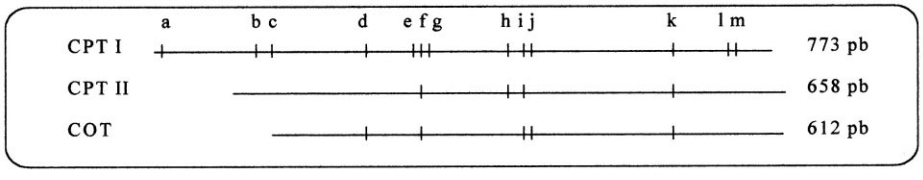
2.4. Immunological techniques

A peptide (EKLLETEGRWKGSEKVR) (residues 344–360) of COT was synthesized as described in [21]. The peptide showed low identity to other carnitine transferases. Generation of anti-COT antibodies was performed as described [4]. γ-Globulins from peptide 344–360 were designated A344. Western blotting analysis was performed as in [22]. *S. cerevisiae* protein extracts were treated with sample buffer and subjected to 10% SDS–polyacrylamide gel electrophoresis. Immunodetection was performed using antiserum A344 (1/100) and the blots were developed with the ECL Western blotting system from Amersham Pharmacia Biotech.

3. Results and discussion

3.1. Analysis of histidines potentially involved in the inhibition of peroxisomal COT by malonyl-CoA

To determine which of the His residues of COT were potentially responsible for the inhibitory action of malonyl-CoA, we examined all the His residues common to seven carnitine acyltransferases (Fig. 1). We postulated that histidines a, b and c cannot be responsible for the binding to malonyl-CoA because they are absent in peroxisomal COT (the first amino acid of the COT begins at position 152 of mitochondrial L-CPT I), provided that both carnitine transferases have conserved the same malonyl-CoA-inhibitable sites. In addition, histidines e, g, h, l and m, which are present in CPT I and not conserved in peroxisomal COT, are not candidates for malonyl-CoA inhibition in COT. Moreover, those His residues present at the same position in all carnitine transferases (histidines f, i and k) cannot be responsible for malonyl-CoA sensitivity because CPT II is insensitive to the inhibitor. According to this reasoning, only two residues, present in the malonyl-CoA-inhibitable enzymes and absent in non-inhibitable carnitine transferases, were candidates for malonyl-CoA inhibition: histidine d, corresponding to peroxisomal COT H131, and histidine j, corresponding to peroxisomal COT H340 from the rat. We then decided to mutate the two His residues presumably involved in binding with malonyl-CoA. Since it has been proposed that histidine i is the amino acid residue responsible for binding and catalysis of its substrate for CPT II [23,24], on the basis of analogy with CPT II we also mutated H327 in COT, to test its role in catalysis.



	a	b	c	d	e	f	g	h	i	j	k	l	m
CPT II human	-	P11	P18	R167	T236	H242	R247	H344	H372	R382	H555	N624	C639
CPT II rat	-	P11	L18	Q167	T236	H242	R247	H344	H372	R382	H555	N624	C639
L-CPT I human	H5	H133	H140	H277	H327	H333	H338	H445	H473	H483	H656	H735	H753
L-CPT I rat	H5	H133	H140	H277	H327	H333	H338	H445	H473	H483	H656	H735	H753
M-CPT I human	H5	H135	H142	H279	H328	H334	H339	H445	H473	H483	H656	H735	H753
M-CPT I rat	H5	H135	H142	H279	H328	H334	H339	H445	H473	H483	H656	H735	H753
COT rat	-	-	-	H131	N178	H191	C196	G299	H327	H340	H506	T577	M595
				*					Δ	*			

Fig. 1. Alignment of conserved histidines in seven carnitine acyltransferases. Sequences were obtained from the GenBank sequence data bank: human CPT II (accession number U09648), rat CPT II (J05470), human L-CPT I (L39211), rat L-CPT I (L07736), human M-CPT I (D64178), rat M-CPT I (D43623) and rat COT (U26033) [15,29–34]. Alignment of sequences was obtained with the PILEUP and PRETTY programs from Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, WI, USA. At the top, the distribution of the 13 conserved histidines is shown. At the bottom, * indicates the conserved histidines in malonyl-CoA-inhibitable enzymes (CPT I and COT), Δ indicates the histidine conserved in all carnitine transferases and presumably involved in the acyl-CoA binding [23,24].

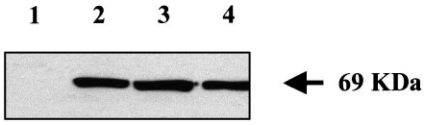


Fig. 2. Immunoblot analysis of recombinant COT variants in *S. cerevisiae*. Samples were prepared and analyzed as described in Section 2. *S. cerevisiae* extracts (10 μg) were separated by SDS–PAGE and subjected to immunoblotting using specific A344 antibodies for COT. Lane 1, extract from *S. cerevisiae* transformed with the empty plasmid; lane 2, COT wild-type; lane 3, mutant H131A/H340A; lane 4, mutant H327A. The arrow indicates the migration position and the molecular mass of rat liver COT.

3.2. Expression of peroxisomal COT in S. cerevisiae and kinetic analysis of the enzyme

The pYES plasmid containing the COT wild-type was expressed in *S. cerevisiae* and the yeast extracts were assayed for COT activity. Only the pellet enriched in peroxisomes exhibited malonyl-CoA sensitivity, as reported by others with rat liver extracts [20,25]. Therefore, we used the membrane fraction for further studies. In cells transformed with the empty plasmid, COT activity was not detected. To rule out the possibility that the various transformed yeast cultures expressed variable amounts of peroxisomal COT we performed an immunoblot analysis. As shown in Fig. 2 all transformed yeast cells expressed a protein with the same molecular mass and in roughly the same proportion per mg of total protein. Thus, *S. cerevisiae* is a good model to study COT activity, as has been found for other carnitine transferases expressed in yeast [12,26–28].

S. cerevisiae cultures transformed with the expression plasmid pYESCOT$^{H131A/H340A}$, containing the double mutant enzyme, hardly grew when the inductor galactose was the carbon source. It was necessary to add glucose first to promote growth, and then galactose to induce the expression of the mutated version of peroxisomal COT.

Yeast-expressed COT activity of the double mutant H131A/H340A was 206 nmol/min/mg protein which was double the value of the wild-type (97 nmol/min/mg protein). Mutant H327A, in which the putative catalytic site had been mutated, lost all enzyme activity. This fact strongly suggests that H327 belongs to the catalytic site, as has also been proposed for CPT II. The specific activities of pYESCOTwt and of the double mutant are high enough to be determined with ease. Rat liver peroxisomal COT was inhibited up to 69% by 200 mM malonyl-CoA, which is consistent with the result (65%) of Ramsay et al. [5]. The malonyl-CoA inhibition of wild-type yeast-expressed COT was also similar (59%). Therefore, the residual activity, not inhibitable, is probably due to the intrinsic characteristics of the enzyme, rather than to the expression system used.

The wild-type and the double mutated version of COT exhibit hyperbolic saturation kinetics in response to varying concentrations of carnitine or decanoyl-CoA (Fig. 3). The calculated apparent K_m values for carnitine in wild-type (172 μM) and the double mutant (106 μM) are similar to rat liver peroxisomes (218 μM). However, the K_m of the two expressed forms for decanoyl-CoA (2.0 and 13.1 μM respectively) were higher than for rat liver peroxisomes (0.2 μM). An increase in the apparent K_m for decanoyl-CoA in the yeast-expressed CPT I was also observed by Prip-Buus et al. [27]. The expression of these proteins in a heterologous system could explain these differences.

3.3. Inhibitory action of malonyl-CoA on the wild-type and mutants of peroxisomal COT expressed in S. cerevisiae

Fig. 4A shows the malonyl-CoA-dependent inhibition of the yeast COT in which the cells had been transformed by the plasmid pYESCOTwt. The inhibitory plots were performed at three different concentrations (5, 50 and 200 μM) of decanoyl-CoA, as substrate. The inhibitory pattern was maximal at the lowest concentration of decanoyl-CoA, showing a competitive effect of the malonyl-CoA towards decanoyl-CoA. Fig. 4B shows the inhibitory pattern of malonyl-CoA on the *S. cerevisiae*-expressed double mutant H131A/H340A of peroxisomal COT. This mutant was not inhibited by malonyl-CoA at any concentration in the range 2–200 μM, nor at any decanoyl-CoA concentration, despite the fact that the enzyme was fully active. It appears that the mutation H/A in

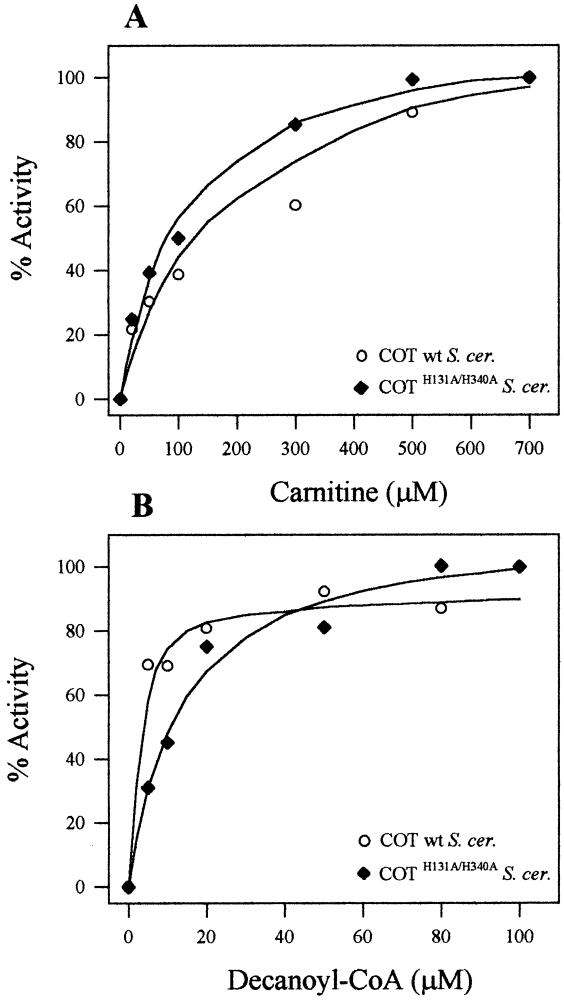


Fig. 3. Dependence of carnitine and decanoyl-CoA of wild-type and mutant COT variants. Yeast extracts of wild-type and the double mutant COT variant were incubated at increasing concentrations of carnitine (A) or decanoyl-CoA (B). When decanoyl-CoA concentration was modified, the L-carnitine concentration was fixed at 400 μM, and when carnitine was modified the decanoyl-CoA concentration was fixed at 50 μM. Data are the mean of three independent experiments.

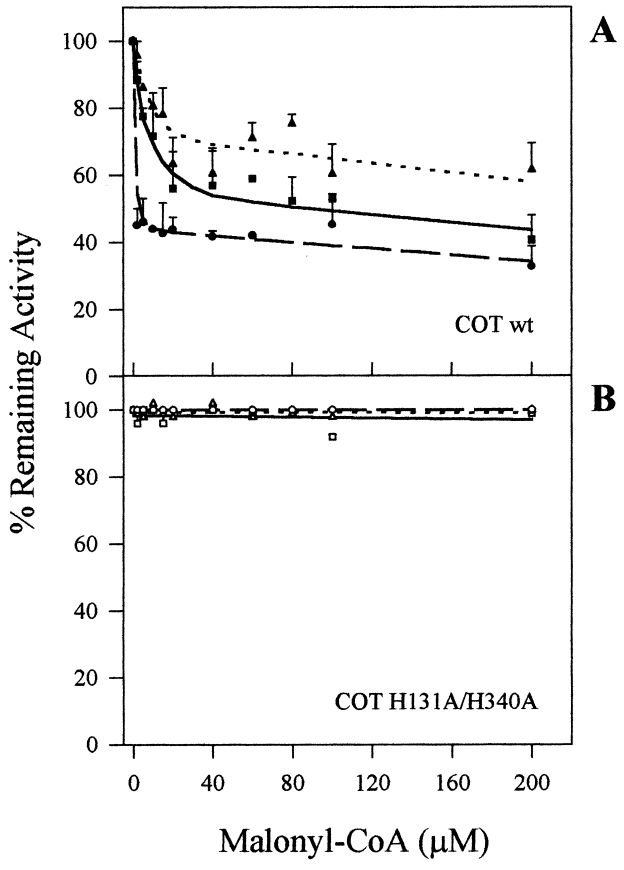


Fig. 4. Effect of malonyl-CoA on the activity of the wild-type and point mutants of COT. *S. cerevisiae* cells were transformed with the cDNAs for the wild-type (A) and the H131A/H340A double mutant (B). Extracts were incubated with several decanoyl-CoA concentrations (5 μM, dashed line; 50 μM, solid line; 200 μM, dotted line) and increasing concentrations of malonyl-CoA. Data are expressed relative to control values in the absence of malonyl-CoA (100%), as the mean of three independent measurements ± S.E.M.

the two putative sites of malonyl-CoA interaction fully abolishes the inhibitory effect, confirming the hypothesis that these are the two COT inhibitory sites of malonyl-CoA. H340, due to its proximity to the catalytic site, may be the site at which malonyl-CoA competes which other CoA esters, as described for other carnitine transferases [5,6,9].

Identification of the histidine residues responsible for the inhibition of carnitine acyltransferases by malonyl-CoA is an important step in elucidating the key to the control of β-oxidation. These results open the way to study whether specific mutations in either carnitine transferase alter the metabolism of fatty acids in several tissues and in the pancreatic β-cell in particular, which may determine the development of diabetes. Studies on this topic are in progress in our laboratory.

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References

[1] Chung, C.-D. and Bieber, L. (1993) J. Biol. Chem. 268, 4519–4524.
[2] Lilly, K., Bugaisky, G.E., Umeda, P.K. and Bieber, L.L. (1990) Arch. Biochem. Biophys. 280, 167–174.
[3] Bieber, L.L., Krahling, J.B., Clarke, P.R., Valkner, K.J. and Tolbert, N.E. (1981) Arch. Biochem. Biophys. 211, 599–604.
[4] Caudevilla, C., Serra, D., Miliar, A., Codony, C., Asins, G., Bach, M. and Hegardt, F.G. (1998) Proc. Natl. Acad. Sci. USA 95, 12185–12190.
[5] Nic a'Bháird, N. and Ramsay, R.R. (1992) Biochem. J. 286, 637–640.
[6] Bird, M.I. and Saggerson, E.D. (1984) Biochem. J. 222, 639–647.
[7] Saggerson, E.D. and Carpenter, C.A. (1981) FEBS Lett. 132, 166–168.
[8] Cook, G.A., Mynatt, R.L. and Kashfi, K. (1994) J. Biol. Chem. 269, 8803–8807.
[9] Kashfi, K., Mynatt, R.L. and Cook, G.A. (1994) Biochim. Biophys. Acta 1212, 245–252.
[10] Mills, S.E., Foster, D.W. and McGarry, J.D. (1984) Biochem. J. 219, 601–608.
[11] Stephens, T.W., Cook, G.A. and Harris, R.A. (1983) Biochem. J. 212, 521–524.
[12] Cohen, I., Kohl, C., McGarry, J.D., Girard, J. and Prip-Buus, C. (1998) J. Biol. Chem. 273, 29896–29904.
[13] Swanson, S.T., Foster, D.W., McGarry, J.D. and Brown, N.F. (1998) Biochem. J. 335, 513–519.
[14] Chatterjee, B., Song, C.S., Kim, J.M. and Roy, A.K. (1988) Biochemistry 27, 9000–9006.
[15] Choi, S.J., Oh, D.H., Song, C.S., Roy, A.K. and Chatterjee, B. (1995) Biochim. Biophys. Acta 1264, 215–222.
[16] Schiestl, R.H. and Gietz, R.D. (1989) Curr. Genet. 16, 339–346.
[17] Sherman, F., Fink, G.R. and Hicks, J.B. (1986) Laboratory Course Manual for Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[18] Datta, A.K. (1995) Nucleic Acids Res. 23, 4530–4531.
[19] Esser, V., Brown, N.F., Cowan, A.T., Foster, D.W. and McGarry, J.D. (1996) J. Biol. Chem. 271, 6972–6977.
[20] Derrick, P. and Ramsay, R.R. (1989) Biochem. J. 262, 801–806.
[21] Marglin, A. and Merrifield, R.B. (1970) Annu. Rev. Biochem. 39, 841–866.
[22] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[23] Brown, N.F., Anderson, R.C., Caplan, S.L., Foster, D.W. and McGarry, J.D. (1994) J. Biol. Chem. 269, 19157–19162.
[24] Schmalix, W. and Bandlow, W. (1993) J. Biol. Chem. 268, 27428–27439.
[25] Broadway, N.M. and Saggerson, E.D. (1997) Biochem. J. 322, 435–440.
[26] Shi, J., Zhu, H., Arvidson, D.N., Cregg, J.M. and Woldegiorgis, G. (1998) Biochemistry 37, 11033–11038.
[27] Prip-Buus, C., Cohen, I., Khol, C., Esser, V., McGarry, J.D. and Girard, J. (1998) FEBS Lett. 429, 173–178.
[28] de Vries, Y., Arvidson, D.N., Waterham, H.R., Cregg, J.M. and Woldegiorgis, G. (1997) Biochemistry 36, 5285–5292.
[29] Finocchiaro, G., Taroni, F., Rocchi, M., Martin, A.L., Colombo, I., Tarelli, G.T. and DiDonato, S. (1991) Proc. Natl. Acad. Sci. USA 88, 661–665.
[30] Woeltje, K.F., Esser, V., Weis, B.C., Sen, A., Cox, W.F., McPhaul, M.J., Slaughter, C.A., Foster, D.W. and McGarry, J.D. (1990) J. Biol. Chem. 265, 10720–10725.
[31] Britton, C.H., Schultz, R.A., Zhang, B., Esser, V., Foster, D.W. and McGarry, J.D. (1995) Proc. Natl. Acad. Sci. USA 92, 1984–1988.
[32] Esser, V., Britton, C.H., Weis, B.C., Foster, D.W. and McGarry, J.D. (1993) J. Biol. Chem. 268, 5817–5822.
[33] Yamazaki, N., Shinohara, Y., Shima, A., Yamanaka, Y. and Terada, H. (1996) Biochim. Biophys. Acta 1307, 157–161.
[34] Yamazaki, N., Shinohara, Y., Shima, A. and Terada, H. (1995) FEBS Lett. 363, 41–45.