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Structurally different rat liver medium-chain acyl CoA dehydrogenases directed by complementary DNAs differing in their 5′-region

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Different forms of rat liver medium-chain acyl CoA dehydrogenase (MCAD) (EC 1.3.99.3) were produced in *Escherichia coli* carrying expression plasmids (pRMCADm-1 ~ 9) differing at the 5′-region of the cDNA. The proteins expressed could be readily extracted from the cells. The protein (~ 44 kDa) directed by pRMCADm-3 showed the highest activity and was readily purified to homogeneity. The purified enzyme contained non-covalently bound FAD and was similar to rat liver mitochondrial enzyme in all respects examined. The purified protein (~ 45 kDa) directed by pRMCADm-1 did not contain FAD and showed no enzymatic activity. Therefore, the leader peptide disturbs the binding of FAD to the apoprotein. The purified protein (~ 40 kDa) directed by pRMCADm-6 did not contain FAD. Thus, the deletion of the NH_2-terminal portion of the apoprotein to some extent results in its inability to combine with FAD.

Introduction

Medium-chain acyl CoA dehydrogenase (MCAD) (also called general acyl-CoA dehydrogenase) (EC 1.3.99.3) is one of the mitochondrial acyl CoA dehydrogenases catalyzing the first step of the β-oxidation cycle in the catabolism of fatty acids. The enzyme is a tetramer of four identical ~ 44 kDa subunits, containing one molecule of non-covalently bound FAD per subunit and catalyzes the dehydrogenation of C_4-C_{12} acyl CoAs with maximal activity toward the C_8 one. In this enzymatic reaction, electron transfer flavoprotein is the obligatory natural electron acceptor which reoxidizes the reduced enzyme [1]. MCAD has been purified to homogeneity from pig kidney [2], pig liver [3], bovine liver [4] and rat liver [5]. Like other nucleus-encoded mitochondrial proteins, the enzyme is synthesized as a precursor having a leader peptide [6–8]. The cDNAs encoding the enzyme were isolated from cDNA libraries of rat liver [9,10] and partial cDNAs from human liver [11,12] and placenta [12]. Recently, we isolated a cDNA for MCAD of nearly full length from human placenta cDNA library [13]. The molecular weights of the precursor and of the mature enzyme of rat liver were estimated to be 46 600 and 43 700 daltons, respectively; thus allowing for a leader peptide of 25 amino acid residues equaling 2900 daltons [9]. The precursor is transferred through an energy-dependent mechanism into mitochondria where its leader peptide is cleaved by protease-processing to produce enzymatically active protein [8]. The leader peptide is presumably indispensable not only for importing and processing steps involving mitochondrial membrane passage, but also for attainment of the three-dimensional structure of the catalytically inactive precursor. From X-ray diffraction study of pig liver MCAD [14], the flavin ring of the coenzyme seems to lie near the NH_2-terminal domain of the apoprotein. Therefore, deletion or modification of the NH_2-terminal portion is expected to give some information on the process of the coenzyme binding for the enzyme activity.

Abbreviations: MCAD, medium-chain acyl CoA dehydrogenase (EC 1.3.99.3); IPTG, isopropyl-β-D-thiogalactopyranoside; pRMCADm, pUC 19 containing rat liver MCAD mutant cDNA; DCIP, dichlorophenolindophenol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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in the present study, by deleting some portions of the 5′-region of the MCAD-encoding cDNA previously isolated [10], the enzymatically active MCAD and the proteins having a part of the leader peptide or lacking the NH_2-terminal region of the mature form were synthesized in *Escherichia coli*. We describe here the properties of these MCAD proteins in relation to the function of this enzyme.

Materials and Methods

Materials

Enzymes for DNA manipulation, plasmid pUC 19 and M13 DNA sequencing kit were purchased from Takara Shuzo (Kyoto, Japan); *E. coli* JM109, from ToYoBo (Tokyo, Japan); BioGel HTP, BioGel A-5m and horseradish peroxidase-conjugated goat anti-rabbit IgG, from Bio-Rad Laboratories (Richmond, CA, U.S.A.); isopropyl-β-D-thiogalactopyranoside (IPTG), from Wako Pure Chemicals (Osaka, Japan); and [α-^{32}P]dCTP, from Amersham International (Buckinghamshire, U.K.). Anti-pig kidney MCAD antiserum was prepared as described previously [7].

Construction of expression plasmid for rat liver MCAD

A nearly full-length cDNA for rat liver MCAD contained 1230 nucleotides of the coding region and 574 nucleotides of the 3′-noncoding region [10]. The cDNA we isolated coincided with that of Matsubara et al. [9] except for the lacking of the first 30 nucleotides of the precursor. The cDNA was inserted into the *Eco*RI site of plasmid pUC 19. Expression plasmids for MCAD were constructed in two different ways: (1) the recombinant plasmid was cleaved with *Hin*d III and *Sal* I, repaired with Klenow fragment and ligated; (2) the recombinant plasmid was cleaved with *Bam* HI and *Sph* I, treated with exonuclease III at 37°C to delete the 5′-region of the cDNA and aliquots were put into the buffer (40 mM sodium acetate (pH 4.5) containing 100 mM NaCl, 2 mM $ZnCl_2$ and 10% glycerol) at 5 s intervals. The deleted plasmids were further digested with mung bean nuclease, repaired with Klenow fragment and ligated. The deletion mutants were designated as the pRMCADm series.

Transformation

E. coli JM109 was used as host strain for expression of rat liver MCAD. Transformation was carried out as described by Hanahan [15].

Colony immunoassay

E. coli JM109 cells transformed with pRMCADm series were spread on LB (Luria-Bertani) agar plates containing 200 μg/ml of ampicillin and 2.5 mM IPTG. The plates were incubated overnight at 37°C, overlaid with a nitrocellulose membrane (BA 85, Schleicher and Schüll, Dassel, F.R.G.) and then incubated at 37°C for 6 h. The membranes were treated with chloroform vapor for 15 min and then incubated overnight at room temperature with 50 mM Tris-HCl (pH 7.5) containing 0.15 M KCl, 20% fetal calf serum, 1 mM DNase, 2 mg/ml of lysozyme and 10 mM $MgCl_2$. The membranes were immunostained by treating with the anti-MCAD antiserum followed by horseradish peroxidase-conjugated goat anti-rabbit IgG as described previously [7].

Growth of bacteria and preparation of cell-free extract

E. coli JM109 cells carrying expression plasmids were grown aerobically at 37°C overnight in 2 ml of LB medium containing 200 μg/ml of ampicillin and 2.5 mM IPTG. The cells were harvested by centrifugation at 8000 × *g* for 10 min, washed once with 50 mM Tris-HCl (pH 7.4) containing 0.15 M KCl and resuspended in 500 μl of 10 mM Tris-HCl (pH 7.4) containing 1 mM EDTA. The suspension was ruptured by multiple sonication (100 watts, 30 s, 6 times), and cell debris were removed by centrifugation at 10000 × *g* for 15 min. The supernatant was used as the cell-free extract.

Purification of rat liver MCAD produced in E. coli carrying the expression plasmid

The cell-free extract (25 ml) prepared as described above from the culture (1 l) was fractionated with ammonium sulfate (20–60% saturation). The fraction was dialyzed against buffer A (10 mM potassium phosphate (pH 7.4) containing 10% glycerol) and applied to a BioGel HTP column (2.5 × 10 cm) previously equilibrated with buffer A. The column was washed with buffer A and eluted with a linear gradient of potassium phosphate (10–400 mM, pH 7.4) containing 10% glycerol. The fraction containing MCAD, which was detected by immunoblotting using anti-pig kidney MCAD antiserum, was added with ammonium sulfate to 80% saturation and the precipitate formed was dissolved in 1 ml of buffer A containing 0.15 M KCl. The sample was subjected to gel filtration on a BioGel A-5m column (1.0 × 90 cm) previously equilibrated with buffer A containing 0.15 M KCl. The fraction containing MCAD was pooled and stored in an ice bath until used.

Assay of MCAD activity

MCAD activity was determined at 32°C by the phenazine methosulfate (1.5 mM)/dichlorophenolindophenol (DCIP) (0.048 mM) method [2] using *n*-octanoyl CoA as substrate. One unit of the enzymatic activity was expressed by the amount of the enzyme that reduced 1 μmol of DCIP per min.

Sequencing of DNA and protein

The DNA sequence of plasmids was determined by the dideoxynucleotide chain termination method [16]

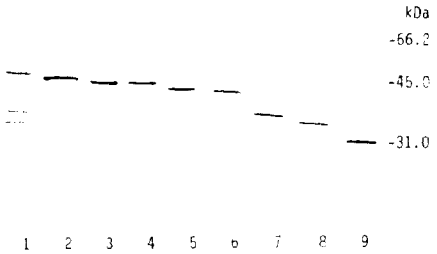

Fig. 1. Immunoblot analysis of cell-free extracts obtained from positive clones. Cell-free extracts from positive clones were subjected to 4–20% SDS-PAGE. Proteins on the gel were transferred to a nitrocellulose membrane and the membrane was immunostained using anti-MCAD antiserum. Number of lane represents the clone carrying the following plasmid: 0, clone carrying pUC 19 alone; 1, pRMCADm-1; 2, pRMCADm-2; 3, pRMCADm-3; 4, pRMCADm-4; 5, pRMCADm-5; 6, pRMCADm-6; 7, pRMCADm-7; 8, pRMCADm-8; 9, pRMCADm-9.

using an M13 sequencing kit. The NH_2-terminal amino acid sequence of the purified MCAD was determined in a gas-phase sequenator (Model 470A, Applied Biosystems, Foster City, CA, U.S.A.). High-performance liquid chromatography was carried out with a Shimadzu model LC-4A apparatus to quantitate the phenylthiohydantoin derivatives produced at each cycle of Edman degradation [17].

Other analyses

Immunoblot analysis was carried out using anti-pig kidney MCAD antibody as described by Towbin et al. [18]. Protein content was determined by the method of Lowry et al. [19] using crystalline bovine serum albumin as standard.

Results

Expression of cDNA encoding rat liver MCAD in E. coli

In order to obtain clones producing MCAD protein, *E. coli* JM109 cultures were transformed with pRMCADm series and colony immunoassay was carried out using anti-MCAD antiserum. Nine clones judged positive by immunoassay were isolated. Fig. 1 shows immunoblot analysis of MCAD proteins produced in *E. coli* carrying pRMCADm-1 ~ 9, indicating the production of the anti-MCAD antibody-reactive proteins with various molecular masses (from 31 to 45 kDa). The extract from pRMCADm-1-bearing bacteria contained two smaller products (35 and 39 kDa) that reacted with the antiserum, in addition to a 45-kDa protein. More than 90% of the protein produced by each clone was recovered in the $10000 \times g$ soluble fraction. These proteins were, however, not produced when the clones were grown in the absence of IPTG. The molecular weights of the proteins were relative to the expected length of the cDNA.

Restriction maps of the cDNAs confirmed the sequences of different length in the 5′-region, corresponding to the various molecular weights. Fig. 2 shows the nucleotide sequences of the 5′-region of the cDNAs, where the downward arrows indicate the junction site between pUC 19 and the cDNAs of pRMCADm-1, 2, 3, 4, 5 and 6.

Octanoyl CoA-dehydrogenating activities of cell-free extracts from these antibody-positive clones were determined. The extract of *E. coli* carrying pRMCADm-3 showed the highest enzymatic activity, whereas those from extracts of *E. coli* carrying pRMCADm-4 and pRMCADm-2 were approx. 90 and 60%, respectively. The extract of *E. coli* bearing pRMCADm-5 showed only marginal activity and the activity in extracts from other clones was below the detection level (data not shown).

Purification of rat liver MCADs produced in E. coli

Among these proteins, those produced in *E. coli* carrying pRMCADm-1, 3 and 6 were purified to compare the characteristics of active and inactive enzymes.

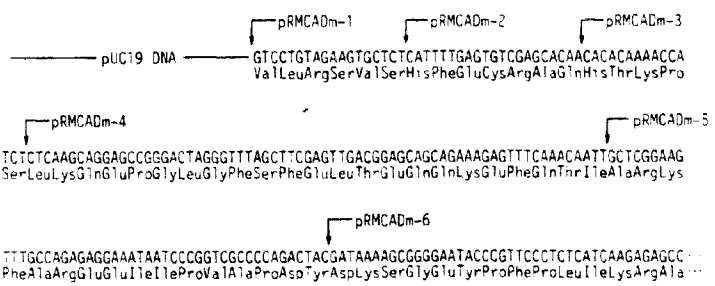

Fig. 2. Nucleotide sequence of 5′-region of expression plasmids pRMCADm-1, 2, 3, 4, 5 and 6. The deduced amino acid sequence is shown under the nucleotide sequence. The downward arrows represent the junction between MCAD gene and pUC 19.

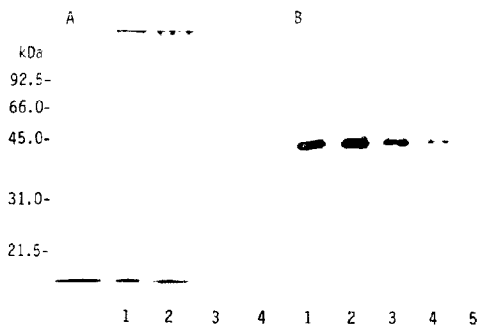

Fig. 3. SDS-PAGE (A) and immunoblot analysis (B) of samples obtained during purification of MCAD produced in *E. coli* carrying pRMCADm-3. Lane 1, cell free extract; 2, ammonium sulfate fraction; 3, BioGel HTP fraction; 4, BioGel A-5m fraction; 5, purified MCAD from rat liver mitochondria [10].

A typical result of the purification procedure for the protein directed by pRMCAD-3 is summarized in Table I. From 485.6 mg protein of the cell-free extract, 3.94 mg of pure MCAD was obtained. Fig. 3 shows the result of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of samples obtained during purification. After hydroxyapatite column chromatography, the protein was almost homogenous; and minor contaminating proteins were removed by gel filtration.

The MCAD proteins of ~40 and ~45 kDa were purified by the same procedure from 216 and 306 mg total protein from *E. coli* harboring pRMCADm-6 and -1, respectively. Their yields were 1.2 mg and 0.24 mg, respectively.

NH_2-terminal sequence of the purified MCAD produced in E. coli carrying pRMCADm-3

Fig. 4 compares the amino acid sequence (B) of the NH_2-terminal region of MCAD deduced from the DNA sequence (A) with that determined by amino acid sequencing of the purified MCAD (C), indicating correctness of the assumed NH_2-terminal sequence.

Properties of the purified MCADs produced in E. coli

The absorption spectrum of the purified MCAD from pRMCADm-3-infected cells exhibited maxima at

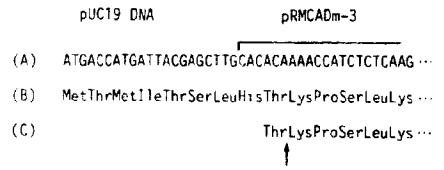

Fig. 4. Nucleotide and deduced amino acid sequences of 5′-region of plasmid pRMCADm-3 and NH_2-terminal sequence of purified MCAD. The upward arrow represents the cleavage site between the leader peptide and the mature enzyme [9]. A, nucleotide sequence; B, deduced amino acid sequence from DNA sequence; C, NH_2-terminal sequence of the purified MCAD determined by chemical analysis.

275, 370, and 445 nm at pH 7.4 (Table II). Fig. 5 shows the visible absorption spectrum, which coincides with that of MCAD purified from rat liver mitochondria. The properties of the enzyme purified from pRMCADm-3-infected cells fairly agree with those of MCAD from rat liver mitochondria (Table II). FAD was released by treating the purified MCAD with 5% trichloroacetic acid, indicating that it is non-covalently

TABLE II

Properties of MCAD purified from E. coli cells carrying pRMCADm-3 and of MCAD from rat liver mitochondria

Property	MCAD produced in *E. coli*/pRMCADm-3	MCAD from rat liver mitochondria [a]
Subunit (kDa)	43 [b]	45
	43.8 [c]	43.7 [d]
Spectral maxima (nm)	275/370/445	271/370/445
Ratio of absorbance maxima (275/370/455) [e]	11.3/0.7/1.0	6.3/0.75/1.0
Specific activity [f] (units/mg protein)	10.3	11.9
K_m (μM)	1.4	4.0
V_{max} (mol DCIP/min per mol enzyme)	32.3	27.2

[a] From Ref. 5.
[b] Estimated by SDS-PAGE in the present study.
[c] Calculated from nucleotide sequence in the present study.
[d] From Ref. 9.
[e] Measured at pH 7.4.
[f] *n*-Octanoyl CoA was used as substrate.

TABLE I

Purification of rat liver MCAD produced in E. coli carrying pRMCADm-3

Procedure	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Cell-free extract [a]	485.6	91.2	0.19	100	1
20–60% $(NH_4)_2SO_4$	314.4	132.8	0.42	146	2.2
BioGel HTP	17.6	62.8	3.57	69	18.8
BioGel A-5m	3.94	40.6	10.30	45	54.2

[a] From 1 l of culture (6 g of cells).

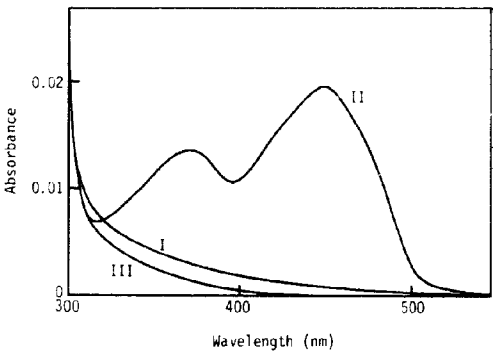

Fig. 5. Visible absorption spectra of purified MCADs produced in *E. coli* carrying pRMCADm-1, -3 and -6. The purified MCAD (0.15 mg/ml) was dialyzed against 50 mM potassium phosphate buffer (pH 7.4) and its spectrum was measured. I, MCAD produced with pRMCADm-1; II, that with pRMCADm-3; III, that with pRMCADm-6.

bound. On the other hand, the purified proteins directed by pRMCADm-1 and pRMCADm-6 did not contain FAD as can be seen in Fig. 5 and showed no enzymatic activity. Also, no activity was observed upon addition of FAD.

Discussion

Precursor proteins including a leader peptide have been reported to form an insoluble material termed 'inclusion body' [20] when synthesized in *E. coli*. On the other hand, precursors without a leader peptide, such as those of cytosolic proteins, are expressed in a soluble form in the bacteria [21,22]. In the present study, the rat liver MCAD proteins synthesized in *E. coli* were recovered in the $10000 \times g$ soluble fraction. This is interpreted as indicating the deletion of amino acids (more than 10 residues) from the leader peptide. This also agrees with the results of Jaussi et al. [23], who reported that the insoluble precursor of chicken mitochondrial aspartate aminotransferase synthesized in *E. coli* was converted to a soluble enzyme upon removal of the leader peptide.

The protein synthesized in pRMCADm-3-infected *E. coli* was expected to be a hybrid protein, since the plasmid contained information for eight extra amino acids of the NH_2-terminal region as shown in Fig. 4. However, in the NH_2-terminal sequence obtained from the purified protein, this extra peptide was found to be absent. This is assumed to be due to the action of bacterial proteinases.

The protein from pRMCADm-3 was purified to homogeneity by a simple procedure using solely hydroxyapatite column chromatography and gel filtration. This protein contained non-covalently bound FAD, and its absorption spectrum in the visible region fairly agreed with those of MCAD from rat liver mitochondria [2–5]. This indicates that FAD in the bacteria is readily available and binds to the apoprotein either during or after the folding of the polypeptide chain, resulting in a stable form. The binding of prosthetic group to apoprotein synthesized in *E. coli* was also reported in the cases of rat liver NADPH-cytochrome *P*-450 oxidoreductase [24] and of rat liver aspartate aminotransferase [22].

The enzymatic properties of the purified MCAD from pRMCADm-3-bearing bacteria were fairly similar to those of the enzyme from rat liver mitochondria. It should be pointed out that activity determinations with MCAD are subject of considerable variation due to difficulties inherent to the assay systems, as noted by several authors [2,25]. The differences to be seen in Table II, in particular that in K_m, might not be significant. However, this problem should await further investigation.

The MCAD purified from the cells carrying pRMCADm-1 did not contain FAD and showed no activity, even in the presence of added FAD. Since the protein directed by pRMCADm-1 has a substantial portion of the leader peptide, the binding of the flavin coenzyme to the apoprotein is probably prevented by the leader peptide.

Further, the MCAD protein purified from pRMCADm-6-bearing cells did not contain any FAD and showed no enzymatic activity, even in the presence of FAD. The crystallographic structure of MCAD from pig liver mitochondria made by Kim and Wu [14] showed that this enzyme consists of three domains. The NH_2-terminal domain is composed of 6 α-helices, the middle one is packed with β-sheets, and the C-terminal one is composed again of 6 α-helices, and the flavin ring lies in the crevice between the first two domains. From the three-dimensional structure, it is apparent that the NH_2-terminal portion of the polypeptide participates in the flavin binding. The deletion of 42 amino acid residues in the protein directed by pRMCADm-6 implies the absence of the first helix of the NH_2-terminal portion [14]. Apparently, the absence of this region results in the failure in forming the functional three-dimensional structure of the mature enzyme, which makes the apoenzyme unable to combine with FAD.

References

1 Crane, F.L., Mii, S., Hauge, J.G., Green, D.E. and Beinert, H. (1956) J. Biol. Chem. 218, 701–716.
2 Thorpe, C., Matthews, R.G. and Williams, C.H. Jr. (1979) Biochemistry 18, 331–337.
3 Kim, J-J. P., Vollmer, S. H. and Frerman, F.E. (1984) J. Biol. Chem. 259, 3318–3319.
4 Dommes, V. and Kunau, W-H. (1984) J. Biol. Chem. 259, 1789–1797.

5 Ikeda, Y., Okamura-Ikeda, K. and Tanaka, K. (1985) J. Biol. Chem. 260, 1313–1325.
6 Mori, M., Matsue, H., Miura, S., Tatibana, M. and Hashimoto, T. (1985) Eur. J. Biochem. 149, 181–186.
7 Inagaki, T., Tsukagoshi, N., Ichihara, C., Ohishi, N., Udaka, S., Ghisla, S. and Yagi, K. (1986) Biochem. Biophys. Res. Commun. 137, 1049–1054.
8 Ikeda, Y., Hale, D.E., Keese, S.M., Cortes, P.M. and Tanaka, K. (1986) Pediatr. Res. 20, 843–847.
9 Matsubara, Y., Kraus, J.P., Ozasa, H., Glassberg, R., Finocchiaro, G., Ikeda, Y., Mole, J., Rosenberg, L.E. and Tanaka, K. (1987) J. Biol. Chem. 262, 10104–10108.
10 Inagaki, T., Ohishi, N., Rasched, I., Frank, R.W., Ghisla, S., Tsukagoshi, N., Udaka, S. and Yagi, K. (1987) Biochem. Int. 15, 1051–1056.
11 Matsubara, Y., Kraus, J.P., Young-Feng, T.L., Francke, U., Rosenberg, L.E., and Tanaka, K. (1986) Proc. Natl. Acad. Sci. USA 83, 6543–6547.
12 Kelly, D.P., Kim, J.J., Billadello, J.J., Hamline, B.L., Chu, T.W. and Strauss, A.W. (1987) Proc. Natl. Acad. Sci. USA 84, 4068–4072.
13 Inagaki, T., Ohishi, N., Bachmann, C., Ghisla, S., Tsukagoshi, N., Udaka, S., and Yagi, K. (1990) J. Clin. Biochem. Nutr. 8, 1–8.
14 Kim, J.-J.P. and Wu, J. (1988) Proc. Natl. Acad. Sci. USA 85, 6677–6681.
15 Hanahan, D. (1985) in DNA cloning (Glover, D.M., ed.), Vol. 1, pp. 109–135, IRL press, Oxford.
16 Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
17 Hankapiller, M.W. and Hood, L.E. (1978) Biochemistry 17, 2124–2133.
18 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
20 Harris, T.J.R. (1983) in Genetic Engineering (Williamson, R., ed.), Vol. 4, pp. 127–185, Academic Press, London.
21 Tanizawa, Y., Kishi, F., Kaneko, T. and Nakazawa, A. (1987) J. Biochem. 101, 1289–1296.
22 Horio, Y., Tanaka, T., Taketoshi, M., Nagashima, F., Tanase, S., Morino, Y. and Wada, H. (1988) J. Biochem. 103, 797–804.
23 Jaussi, R., Behra, R., Giannattasio, S., Flura, T. and Christen, P. (1987) J. Biol. Chem. 262, 12434–12437.
24 Porter, T.D., Wilson, T.E. and Kasper, C.B. (1987) Arch. Biochem. Biophys. 254, 353–367.
25 Pohl, B., Raechle, T. and Ghisla, S. (1986) Eur. J. Biochem. 160, 109–115.