FEBS Letters 441 (1998) 476–479 FEBS 21342

Recombinant bovine lactoperoxidase as a tool to study the heme environment in mammalian peroxidases

Shikiko Watanabe[a,b], Franca Varsalona[b], Yung-Choon Yoo[c], Jean-Paul Guillaume[b], Alex Bollen[b], Keiichi Shimazaki[a], Nicole Moguilevsky[b,*]

[a] *Dairy Science Laboratory, Faculty of Agriculture, Hokkaido University, Sapporo, Japan*
[b] *Applied Genetics, Faculty of Sciences, Université Libre de Bruxelles, 24 rue de l'Industrie, B-1400 Nivelles, Belgium*
[c] *Institute of Immunological Science, Hokkaido University, Sapporo, Japan*

Received 18 September 1998; received in revised form 23 November 1998

Abstract The cDNA encoding bovine lactoperoxidase (LPO) has been expressed in CHO cells. The recombinant LPO was secreted as an enzymatically active single chain molecule presenting two immunoreactive forms of 88 kDa and 82 kDa, differing by their glycosylation. rLPO exhibited the characteristic absorbance spectrum with a Soret peak at 413 nm. Engineering of rLPO into a myeloperoxidase (MPO)-like molecule was attempted by substituting Gln-376 by Met, a residue known to achieve covalent binding with the heme in MPO. However, the resulting bovine LPO mutant failed to acquire the peculiar absorbance spectrum and the chlorinating activity of MPO, underlining the complex nature of interactions in the heme vicinity.

Key words: Recombinant lactoperoxidase; Myeloperoxidase; Heme; Peroxidase activity; Chlorination; Site-directed mutagenesis

1. Introduction

Lactoperoxidase (LPO) is a heme-containing enzyme present in milk, saliva and tears and is part of an antimicrobial defense system, converting thiocyanate to hypothiocyanite in an H_2O_2-dependent reaction. The molecular weight of LPO is approximately 80 kDa and its carbohydrate moiety represents about 10% of the total weight [1,2].

LPO, myeloperoxidase (MPO), eosinophil peroxidase (EPO) and thyroid peroxidase (TPO) belong to the homologous mammalian peroxidase family and share 50–70% identity. An even higher homology can be found among the active site-related residues. These peroxidases have the ability to catalyze the oxidation of halides and pseudohalides such as thiocyanate by hydrogen peroxide to form potent oxidant and bactericidal agents. As the heme is intimately associated with the enzymatic activity, its structure and linkage with neighboring amino acids have been studied in terms of spectral analysis in LPO, EPO and MPO [3–5]. Recently, direct spectroscopic evidence has been given for the presence of two ester linkages between the heme and the protein carbonyl groups in these mammalian peroxidases [5].

MPO is the only member of the family for which the X-ray-derived structure has been described [6,7]; for bovine LPO [8,9], human EPO [10,11] and TPO [12], only primary sequences are known. Based on the MPO X-ray structure, a theoretical model for LPO and EPO has been built, showing evidence supporting the hypothesis of ester linkages between the heme and the apoproteins [13]. The structure and heme binding in MPO have been elucidated and are consistent with a protoporphyrin-IX derivative, linked to the polypeptide chain by two ester linkages with glutamic and aspartic side chains of the residues Glu-408 and Asp-260. An additional covalent sulfonium linkage with the methionine 409 of the MPO polypeptide chain has also been shown [6,7,14,15]. This third covalent linkage with Met-409 is responsible for the peculiar spectroscopic feature of MPO, namely the red shift of the Soret band at 428 nm. MPO is also the only member of the mammalian peroxidase family that can efficiently oxidize chloride to hypochlorite. This peculiar enzymatic property could be conferred by differences in the amino acid environment of the heme, in particular by the presence of Met-409 linkage. All key residues, which constitute the active site wall, on both the proximal and the distal side of the heme, are strictly conserved in LPO, MPO and EPO, the only noteworthy difference being indeed the Met-409 of MPO. Molecular modeling clearly indicates that their location with respect to the heme plan is conserved as well [13].

Previous studies using site-directed mutagenesis of MPO revealed the nature of the bonds linking the heme group to the amino acid backbone and showed the importance of Met-409 for enzymatic activity [15–17] (Fig. 1).

One way to dissect the heme environment in mammalian peroxidases consists of substituting amino acid residues in one type of peroxidase by correspondingly located residues from another type and to follow possible spectral and activity changes. In this respect, the LPO/MPO pair is well suited for these studies since these enzymes clearly differ in absorbance spectrum and type of oxidized halides. Here we report initial work along these lines wherein rLPO was first successfully obtained from engineered CHO cells and shown to display similar activity, spectroscopic and structural features as those of the natural enzyme. In addition, a first amino acid substitution, Gln-376/Met, was performed in an attempt to confer MPO-like absorbance features and chlorinating activity to rLPO. Failure to achieve the objective by a single mutation underlines the complexity of interactions in the heme environment of mammalian peroxidases.

*Corresponding author. Fax: (32) (2) 650 94 72.
E-mail: nmoguil@sga.ulb.ac.be

Abbreviations: r-, recombinant; b-, bovine; LPO, lactoperoxidase; h-, human; MPO, myeloperoxidase; EPO, eosinophil peroxidase; TPO, thyroid peroxidase; CHO, Chinese hamster ovary; OPD, *O*-phenylenediamine; ABTS, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid); ELISA, enzyme linked immunosorbent assay; PCR, polymerase chain reaction

PII: S0014-5793(98)01595-6

2. Materials and methods

bLPO cDNA was amplified from mammary gland cells, using RT-PCR with primers designed from the bLPO cDNA [8]. The length of the amplified fragment covered the full length of bLPO (2295 bp). The sequence analysis was performed in an Applied Biosystems sequencer. The cDNA was introduced into the vector pcDNA3 (Invitrogen) as an *Eco*RI-*Apa*I cassette, leading to the recombinant plasmid pNIV2727. The mutant Gln-376/Met was constructed by the ligation of a 250 bp *Bsm*BI-*Msl*I synthetic adaptor carrying the mutation, leading to the recombinant plasmid pNIV2728. These plasmids were transfected into COS cells, using the calcium phosphate coprecipitation procedure [18], and into CHO cells DG44 *dhfr*$^-$ [19] by electropermeabilization as described [20]. The cells were maintained in αMEM+ supplemented with 5% fetal calf serum, 2 mM L-glutamine, 1% penicillin/streptomycin and selected for resistance to geneticin. The production of rLPO was tested on the supernatant of the clones by ELISA and by measurement of peroxidase activity using ABTS as substrate [21]. After subcloning by limiting dilution, a large-scale cell culture was carried out in a cell factory (Nunc), in the presence of 3% fetal calf serum. The 3–4 day culture supernatants were centrifuged, passed over 0.22 μm filters and stored at 4°C until purification. The purification was performed on a Q-Sepharose fast-flow column and a CM-Sepharose fast-flow column, equilibrated with 20 mM potassium phosphate, pH 7.5 [20]. rLPO was eluted with a liner NaCl gradient (0–450 mM) in the same buffer and collected in 10 ml fractions. Natural bLPO, purified as described [22], was kindly given by Dr. Nakamura, University of Hirosaki, and commercial bLPO was obtained from Sigma. MPO-dependent chlorination activity was measured at 25°C by following the conversion of monochlorodimedon to dichlorodimedon at 290 nm [23]. The covalent binding of heme to rLPO was realized by preincubation of rLPO with 10 μM H_2O_2 as described [24].

Monoclonal antibodies were prepared by fusion of the splenocytes of immunized mice with myeloma cells (P3-X 63. Ag8U1: P3U1). Several monoclonal antibodies were produced in BALB/c mice injected intraperitoneally with the hybridoma cells. Ascitic fluids were collected at 3–4 day intervals, filtered through a 0.45 μm filter and the monoclonal antibody, 1C3-2A, was employed at a 1/10 000 dilution for immunoblotting and 1/7500 for ELISA. Western blots were performed as described [20] and a direct ELISA procedure was developed, using anti-mouse alkaline phosphatase-labelled IgG and *p*-nitrophenylphosphate as substrate. Carbohydrate analysis was carried out using the Glycan Differentiation Kit (Boehringer), with the following lectins: *Galanthus nivalis* agglutinin (GNA); *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA). *N*-Linked carbohydrates were removed by *N*-glycosidase F, high-mannose was removed by endoglycosidase H and fully deglycosylated LPO was prepared by digestion with *N*-glycosidase F, *O*-glycosidase and neuraminidase, following the manufacturer's recommendations (Boehringer). N-terminal amino acid sequence was determined by automated Edman degradation with a Perkin Elmer 492 protein sequencer. The positions of amino acid residues are identified by the sequence alignments in the bovine preprolactoperoxidase and human prepromyeloperoxidase [13].

3. Results and discussion

The sequence of the amplified bLPO cDNA presented three differences with respect to previously reported sequences [8]. However, there was no difference in the deduced amino acid sequence (Table 1). The recombinant plasmid pNIV2727 was transfected into CHO *dhfr*$^-$ cells and geneticin-resistant colonies were selected. After selection and subcloning, one single clone, 9E-3G, which presented the highest peroxidase activity and reactivity in ELISA, was chosen for large-scale production and purification. Since the isoelectric point of bLPO is comparable to that of rMPO, the purification procedure previously used for rMPO was followed [20]. A total of 16 l of spent culture medium was collected, leading after purification to 13 mg of rLPO. After elution from the CM-Sepharose column, the pooled fractions were analyzed on SDS-PAGE and by Western blot, identifying two immunoreactive molecules, a major 88 kDa and a minor 82 kDa species (Fig. 2A,B). The N-terminal amino acid sequences of these two rLPO products were identical and started at residue Asp-101 (Fig. 3). While the cleavage of the propeptide in both 88 kDa and 82 kDa molecules occurred between Arg-100 and Asp-101, this was not the case for purified natural bLPO, nor for commercial bLPO. These molecules were processed by cleavage of the Trp-119/Glu-120 and Thr-115/Ala-116 bonds, respectively (Fig. 3). In fact, the N-terminal end of rLPO was identical to that of the longest bLPO species sequenced by Cals et al. [9]. This extended N-terminal end carries a putative *N*-glycosylation site located at Asn-106.

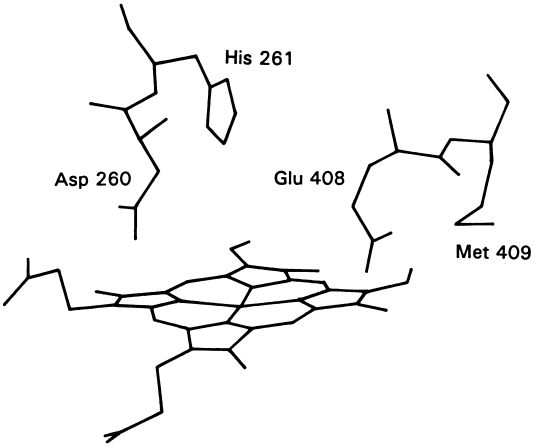

Fig. 1. Heme environment in human MPO, from [17], showing the three covalent bonds to residues Asp-260, Glu-408 and Met-409, based on the 3D model of MPO [6,7].

The enzymatic activity of purified and concentrated rLPO was then measured using ABTS as substrate; it was found to display a lower specific activity than that of the bLPO (184 U/mg versus 337 U/mg). This difference may be accounted for by a lower heme content in the recombinant enzyme. rLPO in its native state [Fe(III)] presents a major absorption peak at 413 nm (Soret peak), likewise bLPO (Table 2). The ratio (RZ) of distinct absorptions at A_{413} and A_{280} was 0.54 for rLPO, as compared to 0.88 for purified bLPO. This lower RZ could reflect, at least in part, a non-covalent state of the prosthetic heme in rLPO, as was previously shown for the rLPO produced in insect cells [24]. To check this possibility we meas-

Table 1
Summary of base changes for published sequences of bovine lactoperoxidase

Base pair position	Present study	Ref. [8]	Ref. [25]	Amino acid position and difference
251	ATT	ATC	ATC	Ile-43, no difference
353	GCC	GCA	GCC	Ala-77, no difference
1598	CCA	CCC	CCC	Pro-492, no difference

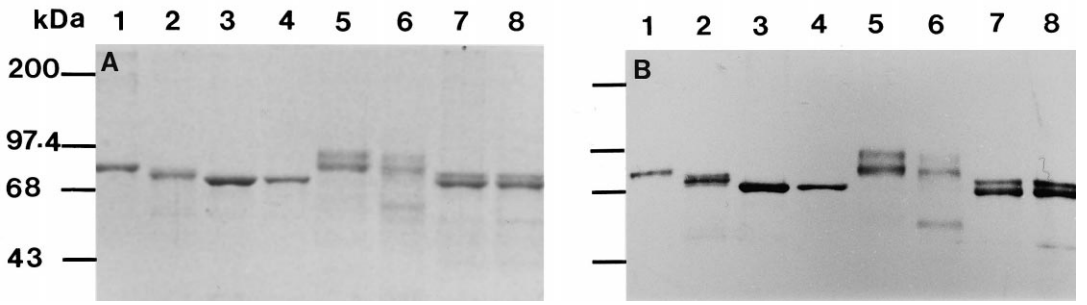

Fig. 2. Carbohydrate analysis of rLPO and bLPO by SDS-PAGE and Western blot of purified LPOs untreated and digested with glycosidases. Proteins were detected by silver staining (A) or by immunoblotting (B). Lane 1: bLPO untreated; lane 2: bLPO digested with endoglycosidase H; lane 3: bLPO digested with *N*-glycosidase F; lane 4: bLPO digested with *N*-glycosidase F, neuraminidase and *O*-glycosidase; lane 5: rLPO untreated; lane 6: rLPO digested with endoglycosidase H; lane 7: rLPO digested with *N*-glycosidase F; lane 8: rLPO digested with *N*-glycosidase F, neuraminidase and *O*-glycosidase. Molecular mass standards are indicated by arrows.

ured the increase in ABTS substrate oxidation by both rLPO and bLPO pretreated with H_2O_2 as described previously [24]. The data showed a 40% increase in the activity of rLPO, whereas there was no significant increase in that of bLPO (Table 2). Apparently, pretreatment or the recombinant enzyme with H_2O_2 increased the covalent binding of the heme to the apoprotein.

Carbohydrate analysis of rLPO and bLPO was performed by sequential digestion of purified proteins using glycosidases, followed by carbohydrate recognition with several specific lectins. After digestion with *N*-glycosidase F, the apparent molecular weight of bLPO (80 kDa) decreased to approximately 70 kDa (Fig. 2A,B). As seen in the same figure, rLPO treated with *N*-glycosidase F shifted in size from the major band of 88 kDa and the minor band of 82 kDa to 73 kDa and 69.5 kDa species. From the shifts in apparent molecular mass, both rLPO and bLPO appear to be *N*-glycosylated to roughly similar extents (16% versus 12.5%). When treated with endoglycosidase H, bLPO displayed a significant mobility change; as seen in Fig. 2A,B, bLPO lost about 8.0 kDa, whereas rLPO decreased only 3 kDa in size. These shifts in apparent molecular mass showed that the natural enzyme had a high mannose content of 9.8%, clearly higher than that of the recombinant enzyme (3.4%). These results also indicated that the high mannose content of natural bLPO reached 78%, whereas it constituted only 20% of the total amount of *N*-glycans in the recombinant enzyme.

Further experiments were performed to determine the presence of *O*-glycosylation in both species. To this end, digestion of the molecules was achieved with *N*-glycosidase F, neuraminidase and *O*-glycosidase. As seen in Fig. 2A,B the size of the rLPO and that of the bLPO did not change, indicating the absence of *O*-linked sugars. Extents of digestion by glycosidases and indications of the nature of the carbohydrate structure in bLPO and rLPO were studied using three lectins: GNA, SNA and MAA (Table 3). rLPO and bLPO reacted with GNA, pointing to the presence of terminal mannose residues on both enzymes, either as high-mannose *N*-glycan chains or as exposed mannose in hybrid chains. The enzymes also reacted with MAA and SNA, revealing terminal *N*-acetylneuraminic acid residues linked α(2-3) and α(2-6) to galactose residues. All these observations were confirmed by the concomitant disappearance of specific lectin binding with the extent of deglycosylation (Table 3).

In view of its overall identity with natural LPO, the recombinant species constitutes a suitable target for molecular engineering. It was attempted to engineer rLPO in its heme environment in order to modify spectral and activity characteristics. The most obvious target was residue Gln-376, which in view of its location appeared to correspond to Met-409 in MPO [13]. Replacement in rLPO of Gln-376 by a methionine residue was thus expected to confer the peculiar absorbance spectrum and chlorinating activity, typical of MPO.

Recombinant plasmid pNIV2728 was therefore introduced into COS cells and the secreted mutant carrying the Gln-376/Met mutation was purified from supernatants of 8 day cell culture. The purified mutant protein migrated on SDS-PAGE as two immunoreactive bands having apparent masses identical as those observed for the wild-type rLPO (82 and 88 kDa). The visible spectrum of the purified mutant was then compared to that of the wild-type rLPO and revealed that the absorption maximum remained at 413 nm, as in the wild-type enzyme, instead of shifting to 428 nm as in MPO (Table 2).

The specific peroxidase activity of the Met-376 mutant was

Table 2
Comparison of properties of bLPO and rLPO, wild-type or carrying the mutation Gln-376/Met

Protein	Soret peak (nm)	Specific peroxidase activity (U/mg)	Specific chlorinating activity (U/mg)
bLPO	413	337	0
rLPO	413	184	0
rLPO+ 10 μM H_2O_2	413	257	0
Met-376 LPO	413	36	0
rMPO	428	69	59

Specific peroxidase activity was measured using ABTS as substrate. Visible spectra were recorded on a Cary 1E spectrophotometer.

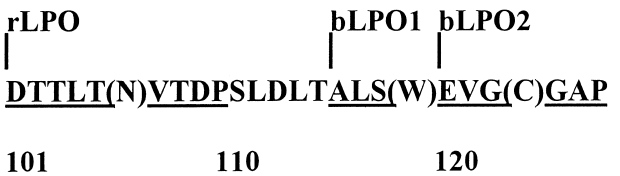

Fig. 3. N-terminal amino acid sequences of purified 82 kDa and 88 kDa rLPO, of purified bLPO (1) and commercial bLPO (2). The sequence spans residues 101–125 of the bLPO amino acid sequence deduced from the cDNA [8]. Underlined residues were identified by automated Edman degradation. Parentheses show unidentified amino acids.

Table 3
Carbohydrate analysis of natural and recombinant lactoperoxidase

Glycosidase treatment	Binding of					
	GNA		SNA		MAA	
	Rec	Nat	Rec	Nat	Rec	Nat
Untreated	+	+	+	+	+	+
Endo H	−	−	+	+	+	+
N-Glyco F	−	−	−	−	−	−
N-Glyco F+*O*-glyco	−	−	−	−	−	−

bLPO and rLPO were treated with several glycosidases and the digested proteins were transferred onto nitrocellulose. Exposed carbohydrates were detected with specific lectins. GNA recognizes terminal mannose, α(1-3), α(1-6) or α(1-2) linked to mannose, thus it is suitable for identifying high-mannose *N*-glycan chains; SNA recognizes sialic acid linked α(2-6) to galactose; MAA recognizes sialic acid linked α(2-3) to galactose. These enzymes are thus suitable for identifying complex, sialylated carbohydrate *N*- or *O*-chains and the type of sialic acid linkage. + and − refer to binding or not of the carbohydrate-specific lectin.

also measured, using ABTS as substrate, and the result showed that the specific activity was lower than for the wild-type species (36.1 versus 184 U/mg). In addition, no chlorinating activity could be detected for the mutant (Table 2). All these results show that the introduction of a methionine instead of a glutamine in rLPO did not modify the spectrum of bLPO, suggesting that the covalent sulfonium linkage of the heme with this methionine was not established in the mutant rLPO.

The failure to engineer MPO-like properties into LPO by a single mutation was disappointing inasmuch as the reverse strategy used previously [15,17], i.e. substituting Met-409 in MPO by Gln, led to the loss of the chlorinating activity, to a red shift of the Soret peak from 428 nm to 413 nm and to the relative decrease in peroxidase activity. Reasons for the failure are not clear at this time but could be found in distortion of the heme plan, which in MPO adopts a bow-like shape [7], or to steric hindrances induced by the mutation. These constraints might have prevented the formation of a sulfonium link with the methionine residue. Nevertheless, the availability of rLPO and the approach here described open the way to a detailed definition of the heme environment in mammalian peroxidases.

Acknowledgements: Supported by Grant 1.5.020.97F from the F.N.R.S. (Fonds National de la Recherche Scientifique). S.W. was the recipient of a grant of the Fondation Brachet (Belgium). Supported by the Food Science Institute Foundation (Ryoshoku-kenkyu-kai, Japan).

References

[1] Carlstrom, A. (1969) Acta Chem. Scand. 23, 171–184.
[2] Mansson-Rahemtulla, B., Rahemtulla, F., Baldone, D.C., Pruitt, K.M. and Hjerpe, A. (1988) Biochemistry 27, 233.
[3] Andersson, L.A., Bylkas, S.A. and Wilson, A.E. (1996) J. Biol. Chem. 271, 3406–3412.
[4] Rae, T.D. and Goff, H.M. (1996) J. Am. Chem. Soc. 118, 2103–2104.
[5] Kooter, I.M., Pierik, A.J., Merkx, M., Averill, B.A., Moguilevsky, N., Bollen, A. and Wever, R. (1997) J. Am. Chem. Soc. 119, 11542–11543.
[6] Zeng, J. and Fenna, R.E. (1992) J. Mol. Biol. 226, 185–207.
[7] Fenna, R., Zeng, J. and Davey, C. (1995) Arch. Biochem. Biophys. 316, 653–656.
[8] Dull, T.J., Uyeda, C., Strosberg, A.D., Nedwin, G. and Seilhamer, J.J. (1990) DNA Cell Biol. 9, 499–509.
[9] Cals, M.-M., Mailliart, P., Brignon, G., Anglade, P. and Dumas, B.R. (1991) Eur. J. Biochem. 198, 733–739.
[10] Ten, R.M., Pease, L.R., Mckean, D.J., Bell, M.P. and Gleich, G.J. (1989) Exp. Med. 169, 1757–1769.
[11] Sakamaki, K., Tomonaga, M., Tsukui, C. and Nagata, S. (1989) J. Biol. Chem. 264, 16828–16836.
[12] Kimura, S., Kotani, T., McBride, O.W., Umeki, K., Hirai, K., Nakayama, T. and Othtaki, S. (1987) Proc. Natl. Acad. Sci. USA 84, 5555–5559.
[13] De Gioia, L., Ghibaudi, E.M., Laurenti, E., Salmona, M. and Ferrari, R.P. (1996) J. Biol. Inorg. Chem. 1, 476–485.
[14] Taylor, K.L., Strobel, F., To You, K., Ram, P., Pohl, J., Woods, A.S. and Kinkade Jr., J.M. (1995) Arch. Biochem. Biophys. 316, 635–642.
[15] Kooter, I.M., Moguilevsky, N., Bollen, A., Sijtsema, N.M., Otto, C. and Wever, R. (1997) J. Biol. Inorg. Chem. 2, 191–197.
[16] Jacquet, A., Deleersnyder, V., Garcia-Quintana, L., Bollen, A. and Moguilevsky, N. (1992) FEBS Lett. 302, 189–191.
[17] Jacquet, A., Garcia-Quintana, L., Deleersnyder, V., Fenna, R., Bollen, A. and Moguilevsky, N. (1994) Biochem. Biophys. Res. Commun. 202, 73–81.
[18] Chen, C. and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752.
[19] Urlaub, G. and Chasin, L.A. (1980) Proc. Natl. Acad. Sci. USA 77, 4216–4220.
[20] Moguilevsky, N., Garcia-Quintana, L., Jacquet, A., Tournay, C., Fabry, L., Piérard, L. and Bollen, A. (1991) Eur. J. Biochem. 197, 605–614.
[21] Venezie, R.D., Jenzano, J.W. and Lundblad, R.L. (1991) J. Clin. Lab. Anal. 5, 57–59.
[22] Morrisson, M. and Hultquist, D.E. (1963) J. Biol. Chem. 238, 2847–2849.
[23] Hager, L.P., Morris, D.R., Brown, F.S. and Eberwein, H. (1966) J. Biol. Chem. 259, 13027–13036.
[24] DePillis, G.D., Ozaki, S., Kuo, J.M., Maltby, D.A. and Ortiz de Montellano, P.R. (1997) J. Biol. Chem. 272, 8857–8860.
[25] Ueda, T., Sakamaki, K., Kuroki, T., Yano, I. and Nagata, S. (1997) Eur. J. Biochem. 243, 32–41.