Characterization of the Maturation of Human Pro-apolipoprotein A-I in an in Vitro Model†

Louise E. Pyle, Dmitri Sviridov,* and Noel H. Fidge

Baker Medical Research Institute, Melbourne, Victoria, 8008, Australia

Received August 25, 2000; Revised Manuscript Received December 8, 2000

ABSTRACT: The reaction conditions and the protein structural features involved in the maturation of pro-apolipoprotein A-I (cleavage of pro-peptide) were investigated in an in vitro model. ProapoA-I, mutants and wild type, were expressed in the PGEX/*E. coli* expression system as fusion proteins with glutathione *S*-transferase (GST). Use of GST−proapoA-I and truncated forms of proapoA-I enabled quantitation of the amount of GST and apoA-I formed as a result of cleavage following incubation with human serum. Deletion of the pro-peptide (GST−apoA-I) resulted in complete inhibition of the reaction. Truncation of proapoA-I to residues 222, 150, 135, and 25 as well as substitution of residues −6, −5, and −4 with alanine did not affect the reaction. Substitution of residues −1, −2, 1, 3, and 4 with alanine either completely blocked or substantially inhibited cleavage of the pro-peptide. The reaction was inhibited by addition of EDTA, *o*-phenanthroline, dithiothreitol, and β-mercaptoethanol and to a lesser extent by *p*-chloromercuriphenylsulfonic acid, but not by leupeptin, *N*-ethylmaleimide, PMSF, pepstatin A, or *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane. Calcium was essential for the activation of the cleavage enzyme, but it had a biphasic effect on the cleavage, activating it at concentrations below 1.5 mM and inhibiting at concentrations above 1.75 mM. Manganese alone was not essential for activation of the enzyme nor did it modify the effect of low concentration of calcium. However, a high concentration of manganese partially reverted the inhibitory effect of a high calcium concentration. Thus, residues within −2 to +4 are involved in forming the cleavage site for the maturation enzyme. The reaction of maturation is inhibited by metalloprotease inhibitors and is dependent upon calcium.

Human apolipoprotein A-I (apoA-I)[1] is the major protein component of high-density lipoprotein (HDL) and a key element of the reverse cholesterol transport pathway, a process which protects against the development of atherosclerosis (*1*). ApoA-I is synthesized in the liver and intestine as a prepro-protein (*2*). The18 amino acid pre-peptide is cleaved during translocation of the protein into the endoplasmic reticulum, and conversion to the mature protein occurs after secretion by removal of the 6 residue pro-peptide in the blood or lymph (*3*). The functional role of the pro-peptide, the requirement for its subsequent cleavage, and the structural features essential for this cleavage are unclear. We (*4, 5*) and others (*6*) have demonstrated that expression of recombinant apoA-I with the pro-peptide deleted impairs secretion and decreases the overall efficiency of the expression of apoA-I, suggesting that apoA-I is synthesized as a pro-protein to aid in intracellular transport of the protein through the cellular apparatus. We have also demonstrated that the presence of pro-peptide impairs interconversion of preβ-HDL into α-HDL (*5*), suggesting a requirement for its cleavage. An apoA-I(P3R) substitution has been recorded, in which the individual was found to have a proapoA-I concentration of the variant protein approximately 3 times the level of the normal protein; however, no detectable disease state was recognizable with this mutation (*7*).

In vivo, the extracellular conversion to the mature form is relatively rapid: proapoA-I constitutes approximately 4−8% of the apoA-I found circulating in the body (*8, 9*) and has a residence time of 0.23 day (*10*). The enzyme responsible for this conversion is unknown. It is secreted by HepG2 cells in vitro (*11*), and decreased proapoA-I converting activity has been identified from individuals with liver cirrhosis and hepatis (*12, 13*), suggesting that the liver plays a role in production of the converting enzyme. In vitro, the half-time for the conversion was found to be on the order of hours, and complete conversion was not achieved within 24 h. The enzyme appears to be a calcium-dependent metalloprotease inhibited by EDTA (*3*).

The requirements for the proapoA-I sequence essential for the effective maturation of proapoA-I are also unknown. The pro-segment of apoA-I is unusual in that it terminates with a Gln-Gln dipeptide rather than a pair of basic amino acids, which are more generally found in vertebrate pro-peptide sequences adjacent to the cleavage site (*14*). When the Gln-Gln pair of the pro-peptide was mutated to Arg-Arg, this alteration did not affect the intracellular transport and

† This work was partly supported by a grant from the National Heart Foundation of Australia (G 96M 4662).

* To whom correspondence should be addressed at the Baker Medical Research Institute, P.O. Box 6492, St. Kilda Rd. Central, Melbourne, Victoria, 8008, Australia. Fax: +61-3-9521-1362; e-mail: Dmitri.Sviridov@Baker.edu.au.

[1] Abbreviations: apoA-I, apolipoprotein A-I; DTT, dithiothreitol; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; GST, glutathione *S*-transferase; NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline (without Ca and Mg); PCMPS, *p*-chloromercuriphenylsulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

10.1021/bi002025g CCC: $20.00 © 2001 American Chemical Society
Published on Web 02/13/2001

secretion of apoA-I from a transfected mouse cell line or its affinity for HDL, but pro-peptide cleavage efficiency was not directly studied in this work (*15*). Stoffel and Niedel (*16*) generated an undecapeptide embracing the human pro-hexapeptide sequence and the first five N-terminal residues of apoAI, but were unable to demonstrate cleavage of this peptide and suggested that the conformation of a specific site may play an important role in cleavage.

In the present paper, we investigated the proapoA-I converting activity using a simple in vitro assay in which a GST−proapoA-I fusion protein is cleaved by serum into GST and apoA-I and the products assessed by SDS−PAGE. We demonstrate that specific residues in positions −2 to + 4 of proapoA-I are involved in forming the cleavage site for the maturation enzyme and that the reaction is dependent upon calcium, is affected by other bivalent cations, and is inhibited by metalloprotease inhibitors.

MATERIALS AND METHODS

Materials. Pepstatin A, leupeptin, and ^{14}C-labeled amino acid mixture (250 μCi) were obtained from ICN (Seven Hills, NSW, Australia). PMSF, *o*-phenanthroline, PCMPS, E-64, NEM, and human thrombin were from Sigma (Castle Hill, NSW, Australia). DTT was from Boehringer Mannheim (Castle Hill, NSW, Australia), and β-mercaptoethanol was from Fluka (Castle Hill, NSW, Australia). ECL Western Blotting detection reagent was from Amersham (Castle Hill, NSW, Australia).

Construction of Recombinant Plasmids and Expression of Fusion Proteins. Utilizing PCR and a full-length apolipoprotein A-I cDNA template, constructs of either proapoA-I or mature apoA-I of varying length were generated by cloning PCR products into a pGEX-KN plasmid (*17*) as described previously (*18*) with the modification that no thrombin cleavage site was encoded in the gene constructs. GST−apoA-I fusion proteins were then generated from an *E. coli* culture as described previously (*18*). Full-length mutants of proapoA-I, substituting [D1A], [QQ−2,−1AA], [RHF−6,−5,−4AAA], and [PP3,4AA], were generated by PCR, and GST fusion proteins were expressed as above. ^{14}C-Labeled GST−proapoA-I fusion protein was synthesized using a modification of the above procedure, wherein the *E. coli* culture was grown in a minimal salts media with the addition of 20% glucose and ^{14}C-labeled amino acids were added to the culture at the time of induction of protein expression. The culture was incubated for a further 2 h and harvested and purified as described previously (*18*). ^{14}C-Labeled GST and proapoA-I were produced using a ^{14}C-labeled GST−proapoA-I fusion protein containing a thrombin cleavage site. The fusion protein was cleaved with thrombin and purified as described (*18*). Proteins were quantitated using the Bradford Protein Assay (*19*).

Assay for Cleavage of GST−ApoA-I Fusion Protein. Ten or fifteen micrograms of GST−apoA-I was added to human serum (final volume 20 μL) and incubated for 0−24 h at 37 °C. The serum−fusion protein mixtures were separated by 12 or 15% SDS−PAGE in a Mini Protean II apparatus (Bio-Rad). Gels containing ^{14}C-labeled proteins were fixed (40% methanol, 10% acetic acid) and dried, and the radioactive proteins were detected and quantitated utilizing a phosphoimager (Fuji BAS 1000). For all other gels, the proteins were transferred to nitrocellulose by electroblotting. The presence of GST and/or apoA-I was detected by Western blotting using either rabbit polyclonal anti-GST antibody or mouse monoclonal antibodies directed against apoA-I: AI-1 epitope residues 28−47 (*20*), AI-3 epitope 140−147 (*20*), or AI-4.1 epitope 211−222 (*21*), followed by anti-rabbit or anti-mouse IgG respectively, coupled to horseradish peroxidase and chemiluminescence development. The resulting films were scanned with a UMAX Power Look II transmission scanner and protein bands quantified using Optimas 6.2 software.

To study the effect of ions on the cleavage activity, aliquots of serum were pretreated with 5 mM EDTA, dialyzed against PBS, and then incubated overnight at 37 °C with GST−proapoA-I forms with the addition of Ca^{2+} and/or Mn^{2+}.

Protease inhibitors when used were added to serum prior to addition of GST−proapoA-I(−6−150), after which 10 μL of serum was incubated with 10 μg of GST−proapoA-I(−6−150) for 16 h at 37 °C.

For analysis of the effect of the mutations on cleavage activity, 10 μg of protein was incubated with 20 μL of whole serum (final volume 30 μL), and incubated for 0, 2, 5, and 17 h, at 37 °C.

RESULTS

Assessment of the Ability of Human Serum To Cleave Propeptide from ProapoA-I. To investigate the requirements for cleavage of proapoA-I, we studied the conversion of fusion protein, GST−proapoA-I, to GST and apoA-I by a factor contained in whole human serum. In the initial experiments, GST−proapoA-I was labeled with [^{14}C]amino acids and incubated for various times with human serum. After separation by SDS−PAGE, gels were exposed to phosphoimager plates, and the products were compared with separately synthesized recombinant [^{14}C]GST and [^{14}C]proapoA-I. The amounts of GST−proapoA-I fusion protein, GST, and apoA-I were then quantitated. In the absence of serum, the GST−proapoA-I fusion protein represented almost all labeled protein (Figure 1A, lane 1). After 24 h incubation with human serum, a significant proportion of the fusion protein was converted into GST and apoA-I (Figure 1A, lanes 2−4). The conversion, however, was not complete even at longer incubation times, indicating that the availability of the substrate is not rate-limiting. The time course of the appearance of [^{14}C]GST (closed symbols) and [^{14}C]apoA-I (open symbols) after incubation of [^{14}C]GST−proapoA-I with human serum is shown in Figure 1B. At short incubation times, the cleavage appears to be linear, but it began to saturate after 5 h incubation (Figure 1B) most likely due to inactivation of the enzyme. It is also apparent from Figure 1B that the appearance of either GST or apoA-I can be utilized to quantitate the cleavage of GST−proapoA-I fusion protein.

Requirements for the Primary Structure of ProapoA-I. To assess whether the cleavage of GST−proapoA-I was in fact measuring the activity of proapoA-I converting enzyme, which produces mature apoA-I, GST−proapoA-I and GST−apoA-I were incubated with human serum for 16 h at 37 °C. Almost no cleavage of GST−apoA-I was observed (Figure 2A, lane 1), while, in contrast, a significant proportion of GST−proapoA-I was cleaved, producing free GST

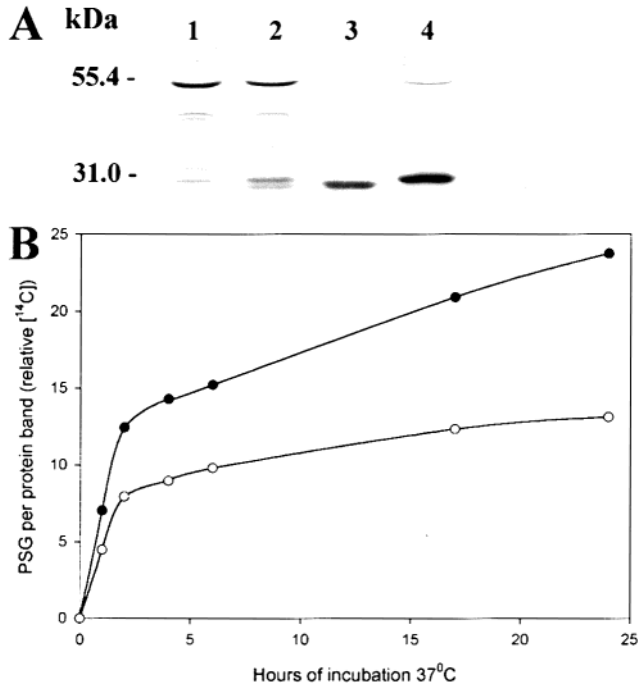


FIGURE 1: Cleavage of GST−proapoA-I into GST and mature apoA-I by serum. Ten micrograms of [^{14}C]GST−proapoA-I was added to 10 μL of whole serum and incubated at 37 °C for 0, 1, 2, 4, 6, 17, and 24 h. For calibration markers, 10 μg of [^{14}C]GST and [^{14}C]proapoA-I was treated as above but without incubation with serum. The samples were diluted to 200 μL, and a 10 μL aliquot was run on 12% SDS−PAGE. A: 12% SDS−PAGE. Lane 1, [^{14}C]-GST−proapoA-I 0 h incubation; lane 2, [^{14}C]GST−proapoA-I 24 h incubation; lane 3, [^{14}C]proapoA-I; lane 4, [^{14}C]GST. B: Time course of cleavage of GST−proapoA-I into GST (●) and mature apoA-I (○).

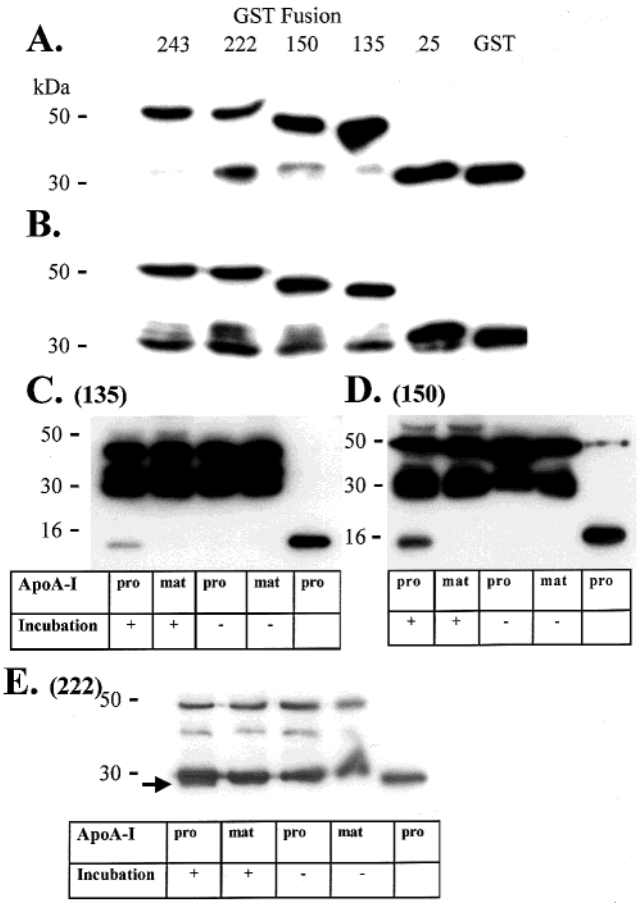


FIGURE 2: Western blot of cleavage of GST−proapoA-I and truncated forms into GST and mature full-length or truncated forms of apoA-I by a factor in human serum. Ten micrograms of GST−apoA-I, pro or mature form, was added to 10 μL of whole serum and incubated at 37 °C for 16 h. The samples were diluted to 200 μL, and a 10 μL aliquot was run on 15% SDS−PAGE and electroblotted to nitrocellulose. Panels A and B: Proteins were identified by probing with rabbit polyclonal anti-GST antibody. A: Lane 1, GST−mature apoA-I(1−243); lane 2, GST−mature apoA-I(1−222); lane 3, GST−mature apoA-I(1−150); lane 4, GST−mature apoA-I(1−135); lane 5, GST−mature apoA-I(1−25); lane 6, GST. B: Lane 1, GST−proapoA-I(−6−243); lane 2, GST−proapoA-I(−6−222); lane 3, GST−proapoA-I(−6−150); lane 4, GST−proapoA-I(−6−135); lane 5, GST−proapoA-I(−6−25); lane 6, GST control. Panels C, D, and E: Proteins were identified by probing with monoclonal anti-apoA-I antibodies. C: Developed with AI-1 monoclonal antibody: Lane 1, GST−proapoA-I(−6−135); lane 2, GST−mature apoA-I(1−135); lane 3, GST−proapoA-I(−6−135) no incubation; lane 4, GST−mature apoA-I(1−135) no incubation; lane 5, proapoA-I(−6−135). D: Developed with AI-3 monoclonal antibody: Lane 1, GST−proapoA-I(−6−150); lane 2, GST−mature apoA-I(1−150); lane 3, GST−proapoA-I(−6−150) no incubation; lane 4, GST−mature apoA-I(1−150) no incubation; lane 5, proapoA-I(−6−150). E: Developed with AI-4.1 monoclonal antibody: Lane 1, GST−proapoA-I(−6−222); lane 2, GST−mature apoA-I(1−222); lane 3, GST−proapoA-I(−6−222) no incubation; lane 4, GST−mature apoA-I(1−222) no incubation; lane 5, proapoA-I(−6−222). Arrow points to the apoA-I(−6−222) band.

(Figure 2B, lane 1). When constructs of GST−pro or mature apoA-I forms truncated to residues 222, 150, 135, and 25 were incubated with serum, all forms of the GST−proapoA-I were cleaved much more efficiently than the mature forms (Figure 2A,B, lanes 2−5), confirming the above result. The fainter bands with the molecular weight slightly higher than GST (Figure 2A, lanes 2−5) are most likely the products of nonspecific degradation of the fusion protein (retained after purification with affinity chromatography).

That the presence of the pro-peptide was essential for activity of the conversion enzyme was further confirmed in experiments where cleavage was assessed by the appearance of apoA-I measured using monoclonal anti-apoA-I antibody. While GST−proapoA-I(−6−135) was cleaved after incubation with the serum (Figure 2C, lane 1), GST−apoA-I(1−135) was not (Figure 2C, lane 2). The same was found for apoA-I truncations to amino acid 150 (Figure 2D, lanes 1, 2) and to amino acid 222 [Figure 2E, lanes 1, 2; arrow points to apoA-I(−6−222) present only in lane 1].

Truncation of GST−proapoA-I to residues 222, 150, 135, and 25 did not eliminate the cleavage (Figure 2B−E), although the absolute rates of the cleavage were not examined. This indicates that the presence of a large segment of proapoA-I, shown here to be at least residues 26−243, is not essential for cleavage of the pro-peptide.

To further investigate the requirements for the residues contained around the proapoA-I cleavage site, several residues were converted to alanine residues. Converting residue number 1 of the mature apoA-I protein, aspartic acid, to alanine [proapoA-I(D1A)] almost completely blocked cleavage (Figure 3A, lane 2, Figure 3B). Altering the two proline residues at positions 3 and 4 [proapoA-I(PP3,4AA)] appears to substantially slow the reaction, although it was not completely inhibited (Figure 3A, lane 3, Figure 3B). Mutating the two glutamine residues at positions −2 and −1 [proapoA-I(QQ−2,−1AA)] slowed the rate of cleavage,

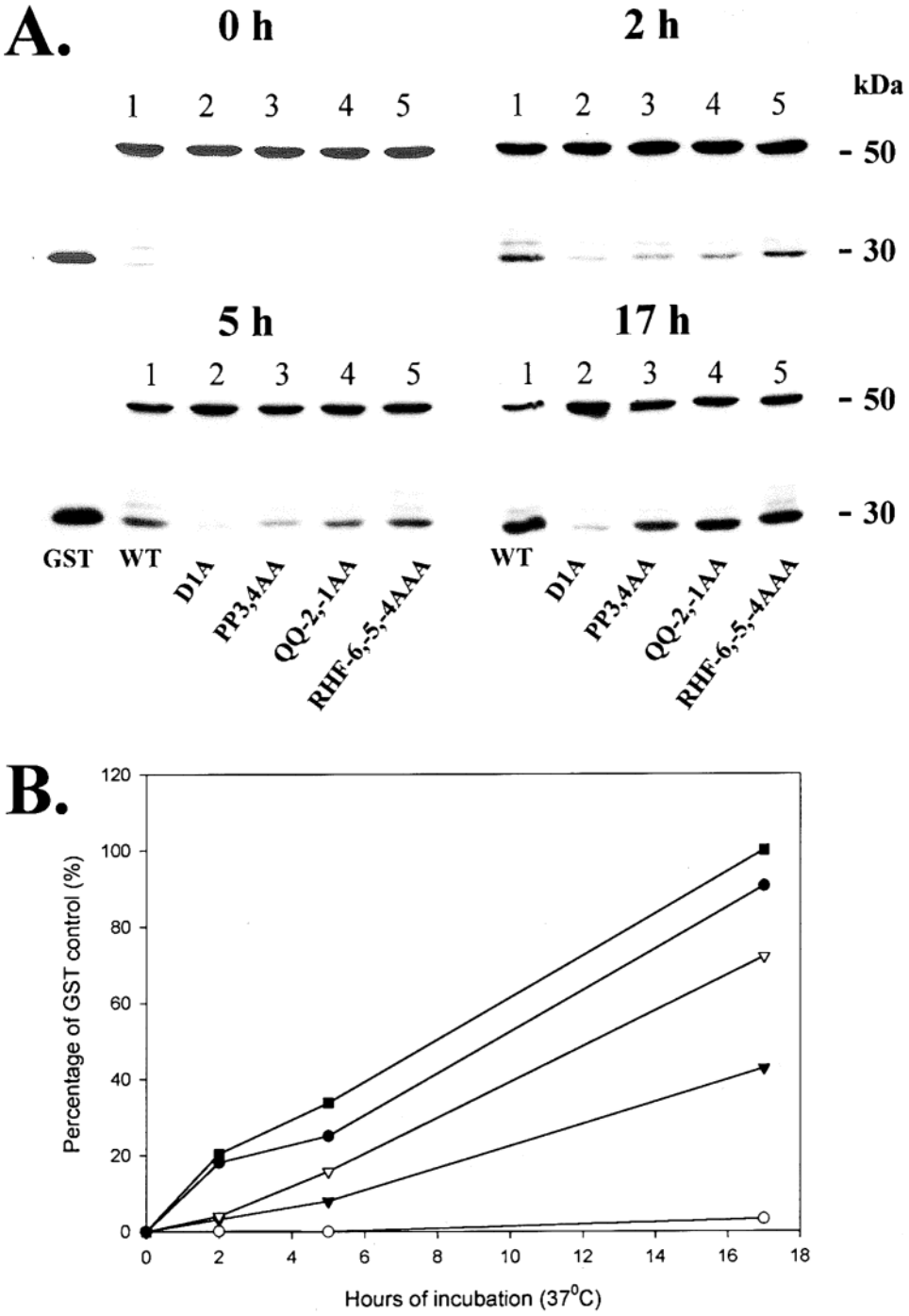


FIGURE 3: Western blot analysis and time course of cleavage of GST−proapoA-I mutants. Ten micrograms of GST−proapoA-I, mutant or wild-type form, was added to 20 μL of whole serum and incubated for 0, 2, 5, and 17 h at 37 °C. The samples were diluted to 400 μL, and a 5 μL aliquot was run on 15% SDS−PAGE and electroblotted to nitrocellulose. Proteins were identified by probing with a rabbit polyclonal anti-GST antibody. A: Western blots: Lane 1, GST−proapoA-I; lane 2, GST−proapoA-I(D1A); lane 3, GST−proapoA-I(PP3,4AA); lane 4, GST−proapoAI(QQ−2,−1AA); lane 5, GST−proapoA-I(RHF−6,−5,−4AAA). B: Time course of GST−proapoA-I cleavage. GST−proapoA-I (●), GST−proapoA-I(D1A) (○), GST−proapoA-I(PP3,4AA) (▼), GST−proapoAI(QQ−2,−1AA) (▽), and GST−proapoA-I(RHF−6,−5,−4AAA) (■).

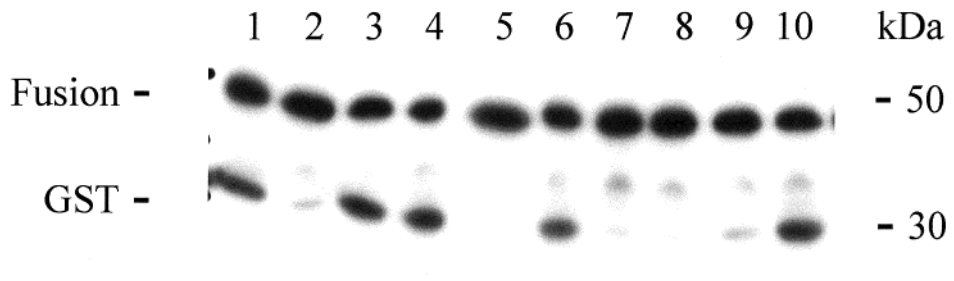
but to a lesser degree than proapoA-I(PP3,4AA)(Figure 3A, lane 4, Figure 3B). Mutating the residues at positions −6, −5, and −4 of the pro-peptide, that is, arginine, histidine, and phenylalanine, to alanine [proapoA-I(RHF−6,−5,−4AAA)] had no substantial effect on the converting activity (Figure 3A, lane 5, Figure 3B). Thus, residues within the region −2 to +4 apparently form a cleavage site for the enzyme responsible for the maturation of proapoA-I in the serum.

Sensitivity to Protease Inhibitors. To compare the properties of proapoA-I maturation in our in vitro model with the properties of the proapoA-I converting enzyme described by Edelstein et al. (*3*, *11*), we assessed the effect of protease inhibitors on GST−proapoA-I cleavage. In these experiments, we used the truncated form, GST−proapoA-I(−6−150), to avoid the occurrence of degradative cleavage of the C-terminus of apoA-I, which may be influenced by protease inhibitors. Formation of free GST after incubation of the

Table 1: Effect of Protease Inhibitors on Cleavage of GST−ProapoA-I(−6−150) by Serum[a]

protease inhibitor	concentration	% inhibition	protease inhibitor	concentration	% inhibition
pepstatin	50 μM	0	E-64	2.5 μM	0
leupeptin	0.5 mM	0	E-64	5.0 μM	8
PMSF	0.5 mM	0	E-64	10.0 μM	0
EDTA	0.5 mM	16	DTT	0.5 mM	76
EDTA	2.5 mM	87	β-mercaptoethanol	0.5 mM	50
EDTA	5.0 mM	85	PCMPS	0.5 mM	0
o-phenanthroline	0.5 mM	33	PCMPS	2.5 mM	0
o-phenanthroline	2.5 mM	99	PCMPS	5.0 mM	36
o-phenanthroline	5.0 mM	93	*N*-ethylmaleimide	2.5 mM	0

[a] Cleavage assay was conducted as described under Materials and Methods, and Western blots were quantified using Optimas 6.2. Inhibition tests were done on 50% serum. % inhibition = (density of GST protein band formed without inhibitor − density of GST protein band with inhibitor)/(density of GST protein band formed without inhibitor − density of GST protein band present in nonincubated sample) × 100.



Serum	WS	WS	WS	-	-	-	DS	DS	DS	DS
EDTA (mM)	0	5	5	0	0	0	0	5	5	5
Ca (mM)	0	0	10	0	0	0	0	0	10	0
>10 kDa	-	-	-	+	-	+	-	-	-	-
<10 kDa	-	-	-	-	+	+	-	-	-	+

FIGURE 4: Western blot analysis of cleavage of GST−proapoA-I(−6−135): Effect of EDTA and dependence on the concentration of Ca^{2+}. Ten micrograms of GST−proapoA-I(−6−135) was added to 17.9 μL of whole serum (WS) or serum which had been dialyzed into PBS without Ca^{2+} and Mg^{2+} (DS) and incubated at 37 °C for 16 h. Alternatively, 500 μL of serum was separated into a fraction of molecular mass greater than 10 kDa (>10 kDa) or less than 10 kDa (<10 kDa) by spinning in a Microcon 10 for 30 min at 4 °C. Ten micrograms of GST−proapoA-I(−6−135) was added to 12 μL from 330 μL of the >10 kDa fraction or to 17.9 μL from 170 μL of the <10 kDa fraction, final volume 20 μL, and incubated at 37 °C for 16 h. 5 mM EDTA or 10 mM Ca^{2+} was added to the serum before incubation as indicated. When DS was used, EDTA was added when indicated prior to dialysis. The samples were diluted to 100 μL, and a 5 μL aliquot was run on 15% SDS−PAGE and electroblotted to nitrocellulose. Proteins were identified by probing with a rabbit polyclonal anti-GST antibody. Lane 1, WS; lane 2, WS + 5 mM EDTA; lane 3, WS + 5 mM EDTA + 10 mM Ca^{2+}; lane 4, >10 kDa fraction; lane 5, <10 kDa fraction; lane 6, >10 kDa fraction + <10 kDa fraction; lane 7, DS; lane 8, DS + 5 mM EDTA; lane 9, DS + 5 mM EDTA + 10 mM Ca^{2+}; lane 10, DS + 5 mM EDTA + <10 kDa fraction.

fusion protein with human serum was assessed using quantitative Western blotting (see Materials and Methods). The agents that most inhibited serum enzyme activity were EDTA, *o*-phenanthroline, DTT, and β-mercaptoethanol, and to a lesser extent PCMPS, a thiol inhibitor which is more soluble than *p*-chloromercuribenzoate (Table 1). Neither leupeptin, NEM, PMSF, pepstatin A, nor E-64 at the concentrations used had any significant effect on cleavage of the fusion protein (Table 1). These results are consistent with the findings of Edelstein et al. (*3, 11*) suggesting that the cleavage activity monitored in our experiments and that in the previous reports (*3, 11*) are similar in nature.

Requirements for Bivalent Cations. The cleavage of GST−proapoA-I was inhibited by treatment of serum with 5 mM EDTA (Figure 4, lanes 1, 2), but the inhibition can be overcome by addition of 10 mM Ca^{2+} (Figure 4, lanes 1−3), indicating a requirement for Ca^{2+} in the cleavage activity. However, when serum to which 5 mM EDTA was added was dialyzed to remove free ions and EDTA complexes, activity was not recovered by subsequent addition of 10 mM Ca^{2+} (Figure 4, lanes 7−9). This indicated either that Ca^{2+} is essential but not sufficient for the GST−proapoA-I cleavage or that an optimal concentration of Ca^{2+} is required for the activity of the enzyme. To further investigate the requirement for a low molecular weight substance(s) for the activity of the maturation enzyme, serum was separated in a Microcon device into two fractions: >10 kDa and <10 kDa. Most of the cleavage activity was found in the >10 kDa fraction (Figure 4, lane 4), and no cleavage activity was found in the <10 kDa fraction (Figure 4, lane 5). Fast separation of plasma components on Microcon would remove a percentage of the low molecular weight components (e.g., peptides) from the >10 kDa fraction; however, a significant proportion of Ca^{2+} and possibly other ions will remain bound to the plasma proteins or free in the plasma as separation is not complete, potentially satisfying the requirements of the maturation enzyme. When the two fractions were recombined, the cleavage activity was fully restored (Figure 4, lane 6), indicting lack of the low molecular weight inhibitor in the <10 kDa fraction. When the <10 kDa fraction was added

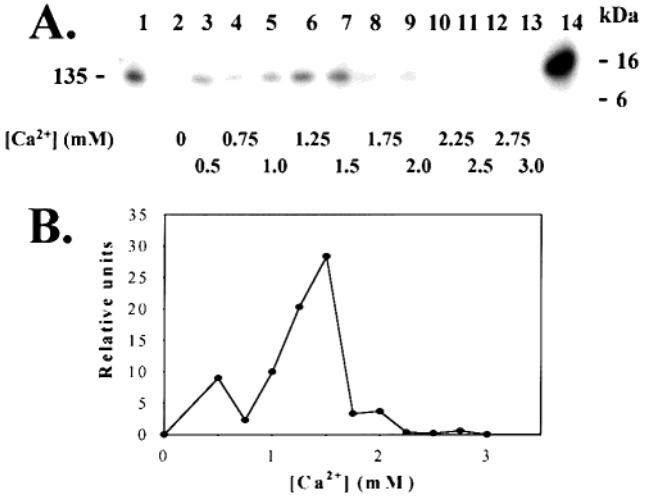


FIGURE 5: Dependence of GST−proapoA-I(−6−135) cleavage by EDTA-treated serum (ES) on the concentration of Ca^{2+}. Five millimolar EDTA was added to whole serum and the serum dialyzed by several changes into PBS at 4 °C (ES). Then 9.5 μg of GST−proapoA-I(−6−135) was added to 17 μL of ES, final volume 20 μL, and incubated for 16 h at 37 °C. Indicated concentrations of Ca^{2+} were added to the reaction mixture before the incubation. The samples were diluted to 100 μL, and a 5 μL aliquot was run on 15% SDS−PAGE and electroblotted to nitrocellulose. Proteins were identified by probing with monoclonal anti-apoA-I antibody. A: Western blotting: Lane 1, whole serum (WS); lane 2, ES + 0 mM Ca^{2+}; lane 3, ES + 0.5 mM Ca^{2+}; lane 4, ES + 0.75 mM Ca^{2+}; lane 5, ES + 1.00 mM Ca^{2+}; lane 6, ES + 1.25 mM Ca^{2+}; lane 7, ES + 1.50 mM Ca^{2+}; lane 8, ES + 1.75 mM Ca^{2+}; lane 9, ES + 2.00 mM Ca^{2+}; lane 10, ES + 2.25 mM Ca^{2+}; lane 11, ES + 2.5 mM Ca^{2+}; lane 12, ES + 2.75 mM Ca^{2+}; lane 13, ES + 3.00 mM Ca^{2+}; lane 14, proapoA-I(−6−135) control. B: Dose dependence of the effect of Ca^{2+} on the activation of GST−proapoA-I(−6−135) cleavage.

to the serum, which had been treated with EDTA and then dialyzed, cleavage activity was also restored (Figure 4, lane 10), which rules out the possibility that removal of Ca^{2+} causes an irreversible conformation change of the enzyme, affecting its activity. Combined, these data indicate that an optimal physiological level of Ca^{2+}, or another ion, rather than a low molecular weight cofactor, is most likely required for the activity of the cleavage enzyme. They also show that the replacement of a high concentration of Ca^{2+} in the absence of EDTA may become inhibitory.

To confirm further the lack of requirement for a low molecular weight cofactor and to determine the exact optimal concentration of Ca^{2+}, dose-dependence experiments were conducted in which GST−proapoA-I(−6−135) was used as substrate and the appearance of apoA-I(1−135) was monitored by Western blotting (see Materials and Methods). The difference in molecular weight between full-length apoA-I and apoA-I(1−135) enabled distinction between the cleavage product and serum apoA-I. EDTA (5 mM) was added to human serum followed by dialysis to remove both free EDTA and bivalent cations. Serum was then incubated with GST−proapoA-I(−6−135) in the presence of 0.5−3 mM Ca^{2+}. Activation of the cleavage enzyme occurred between 0.5 and 1.5 mM Ca^{2+} (Figure 5A, lanes 1−7, Figure 5B); however, higher Ca^{2+} concentrations from 1.75 to 3.0 mM inhibited the cleavage (Figure 5A, lanes 1, 8−13, Figure 5B). Extrapolation of these concentrations to whole human serum indicates that Ca^{2+} becomes inhibitory above approximately 2.4 mM, which is the physiological level of Ca^{2+} in human serum.

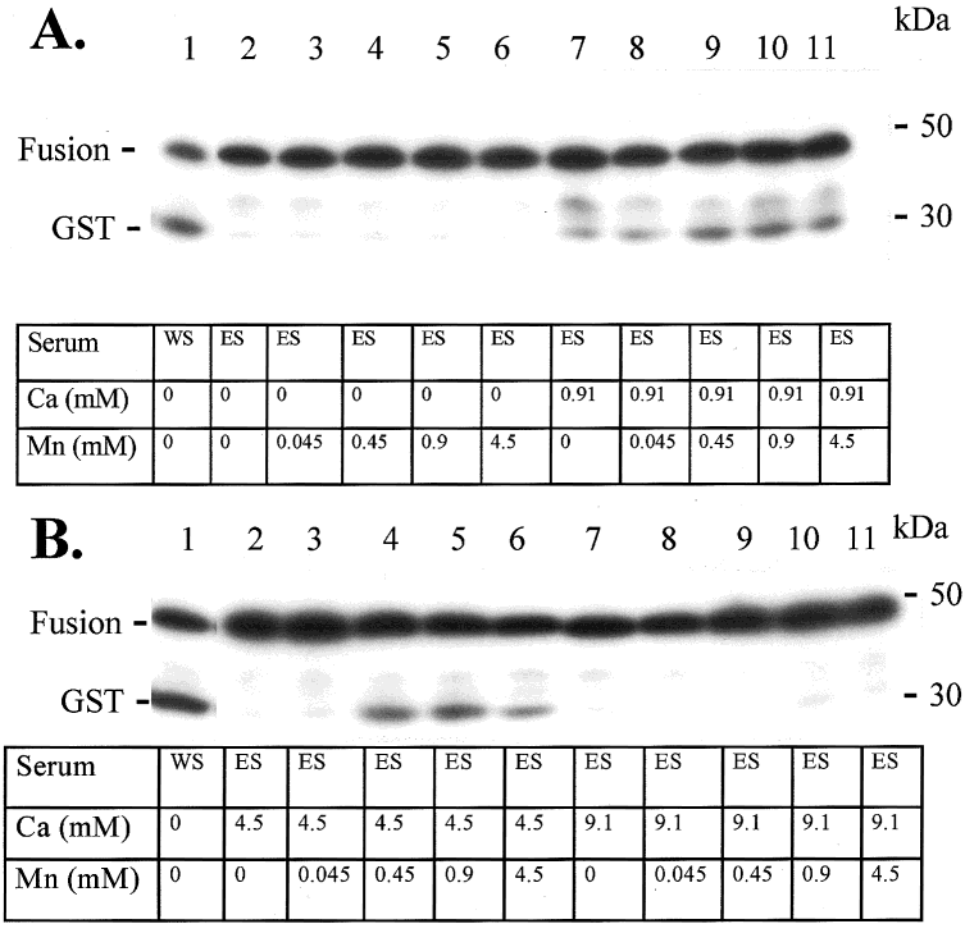
To investigate the requirement for another bivalent cation, Mn^{2+}, similar experiments were conducted as described for the Ca^{2+} dependence; the appearance of free GST was monitored in these experiments. In the absence of Ca^{2+}, addition of Mn^{2+} in concentrations raging from 45 μM to 4.5 mM failed to activate the enzyme (Figure 6A, lanes 1−6), indicating that Mn^{2+} cannot substitute for Ca^{2+} in the reaction. In the presence of the activating concentration of Ca^{2+} (0.91 mM), addition of Mn^{2+} had little effect on the cleavage (Figure 6A, lanes 7−11). However, when Ca^{2+} was added at a concentration which inhibits the cleavage (4.5 mM), addition of increasing concentrations of Mn^{2+} overcame the inhibitory effect of Ca^{2+} and activated the enzyme (Figure 6B, lanes 1−6). When the concentration of Ca^{2+} was raised even further (9.1 mM), the cleavage was effectively blocked even in the presence of high concentrations of Mn^{2+}-(Figure 6B, lanes 7−11).

DISCUSSION

Maturation of proapoA-I (cleavage of pro-peptide) is a very effective process occurring in the blood. The requirements for maturation are unclear: proapoA-I and mature apoA-I have very similar properties when investigated in vitro. The enzyme responsible for the maturation is yet to be identified, and very little is known about the properties of the reaction.

In the present paper, we describe a new in vitro model which facilitates the study of maturation of proapoA-I. The model utilizes a recombinant protein substrate comprising a fusion between GST and proapoA-I. When exposed to human serum, the pro-peptide, still coupled to the GST, is released from mature apoA-I. The fusion protein can be biosynthetically labeled and labeled GST−proapoA-I, GST, and apoA-I determined after SDS−PAGE, to assess the rate of the reaction. Alternatively, unlabeled fusion protein can be used and the amount of released free GST assessed by quantitative Western blotting. Unlabeled wild-type apoA-I cannot be distinguished from endogenous serum apoA-I; however, if a truncated form of recombinant proapoA-I is used, truncated apoA-I released can be clearly distinguished by Western blotting and used to quantify the rate of the reaction. Maturation activity was easily detected in human serum using this model, and all three methods gave very similar results.

This novel approach has resulted in new findings about the nature of the cleavage site and some properties of the cleavage reaction. First, deletion of the pro-peptide (in effect, substitution of the pro-peptide with the C-terminus of the GST component, that is, Gly-Gly-Gly-Gly-Arg-Ser) abolished the cleavage, demonstrating that at least part of the sequence of the pro-peptide is required for the reaction. Second, truncation of apoA-I from the C-terminus up to amino acid 25 did not affect cleavage, indicating that most of the apoA-I sequence is not required for the reaction, e.g., ruling out its involvement in ensuring a correct conformation of the cleavage site. Third, point mutations in close proximity to the cleavage site demonstrated that amino acids within the range −2 to +4 are essential for cleavage and most likely form the cleavage site. The rate of conversion was most significantly affected by the substitution of aspartic acid at



Serum	WS	ES	ES	ES	ES	ES	ES	ES	ES	ES	ES
Ca (mM)	0	0	0	0	0	0	0.91	0.91	0.91	0.91	0.91
Mn (mM)	0	0	0.045	0.45	0.9	4.5	0	0.045	0.45	0.9	4.5

Serum	WS	ES	ES	ES	ES	ES	ES	ES	ES	ES	ES
Ca (mM)	0	4.5	4.5	4.5	4.5	4.5	9.1	9.1	9.1	9.1	9.1
Mn (mM)	0	0	0.045	0.45	0.9	4.5	0	0.045	0.45	0.9	4.5

FIGURE 6: Cleavage of GST−proapoA-I(−6−135) in EDTA-treated serum. Effect of Mn^{2+} on Ca^{2+}. Five millimolar EDTA was added to whole serum and the serum dialyzed by several changes into PBS at 4 °C. Ten micrograms of GST−proapoA-I(−6−135) was added to 18 μL of ES with the addition of the indicated concentrations of Ca^{2+} and Mn^{2+} and incubated for 16 h at 37 °C. The samples were diluted to 100 μL, and a 5 μL aliquot was run on 15% SDS−PAGE and electroblotted to nitrocellulose. Proteins were identified by probing with a rabbit polyclonal anti-GST antibody. WS, whole serum; ES, EDTA-treated serum. Concentrations of Ca^{2+} and Mn^{2+} are shown on the figure. A: Stimulating concentrations of Ca^{2+}. B: Inhibiting concentrations of Ca^{2+}.

residue 1 with alanine, indicating the primary importance of this residue for conversion. The simultaneous substitution of two prolines at residues 3 and 4 also substantially reduced the reaction rate. Aspartic acid at residue 1 and proline at residue 3 are conserved in the bovine (*22*), chicken (*23*), dog (*24*), human (*2*), macaque (*25*), rabbit (*26*), and rat (*27*) proteins, strengthening the importance of these residues. Mutations within the pro-peptide had a lesser effect on the converting activity compared with the mutations in the N-terminus of mature apoA-I. While the bovine, human, and macaque pro-sequences consist of Arg-His-Phe-Trp-Gln-Gln, that of chicken is Arg-Ser-Phe-Trp-Gln-His, and rat Trp-Glu-Phe-Trp-Gln-Gln, indicating some allowable variability in the pro-sequence. Mutating the first three residues of the human sequence, Arg-His-Phe, did not appear to affect the converting activity at all; however, there was some reduction in the reaction rate when the Gln-Gln pair was altered.

Dependence of the reaction on bivalent cations and its susceptibility to protease inhibitors were also studied. Edelstein et al. (*3*, *11*) reported previously that the enzyme is likely to be a metalloprotease, dependent upon calcium and inhibited by certain thiol protease inhibitors. Although our results confirm this finding, they also indicate that high levels of calcium inhibit the converting activity. The optimal Ca^{2+} concentration seems to be around 1.5 mM. Considering that about 40% of calcium in plasma (total concentration 2.4 mM) is bound to proteins and peptides, this would translate into the concentration of free Ca^{2+} in plasma being optimal for the activity of the enzyme. Manganese, although not essential for the reaction, can reverse the inhibitory effect of high concentrations of calcium.

In conclusion, a new model to study maturation of proapoA-I is described. Using this model, we demonstrated that residues within the range −2 to + 4 of proapoA-I are involved in forming a cleavage site for the maturation enzyme and that the reaction is dependent upon calcium, may be regulated by manganese, and is blocked by metalloprotease inhibitors.

REFERENCES

1. Fielding, C. J., and Fielding, P. E. (1995) *J. Lipid Res. 36*, 211−228.
2. Law, S. W., and Brewer, H. J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 66−70.
3. Edelstein, C., Gordon, J. I., Toscas, K., Sims, H. F., Strauss, A. W., and Scanu, A. M. (1983) *J. Biol. Chem. 258*, 11430−11433.
4. Pyle, L. E., Fidge, N. H., Barton, P. A., Luong, A., and Sviridov, D. (1997) *Anal. Biochem. 253*, 253−258.
5. Sviridov, D., Pyle, L. E., Jauhiainen, M., Ehnholm, C., and Fidge, N. H. (2000) *J. Lipid Res. 41*, 1872−1882.

6. McLeod, R. S., Robbins, C., Burns, A., Yao, Z., and Pritchard, P. H. (1994) *Biochem. J. 302*, 641−648.
7. von Eckardstein, A., Funke, H., Henke, A., Altland, K., Benninghoven, A., and Assmann, G. (1989) *J. Clin. Invest. 84*, 1722−1730.
8. Contiero, E., Ferrari, R., Vaselli, G. M., and Folin, M. (1997) *Electrophoresis 18*, 122−126.
9. Brouillette, C. G., and Anantharamaiah, G. M. (1995) *Biochim. Biophys. Acta 1256*, 103−129.
10. Bojanovski, D., Gregg, R. E., Ghiselli, G., Schaefer, E. J., Light, J. A., and Brewer, H. B., Jr. (1985) *J. Lipid Res. 26*, 185−193.
11. Edelstein, C., Kaiser, M., Piras, G., and Scanu, A. M. (1988) *Arch. Biochem. Biophys. 267*, 23−30.
12. Suehiro, T., Yamamoto, M., Yoshida, K., and Ohno, F. (1993) *Clin. Chem. 39*, 60−65.
13. Isobe, H., Sakai, H., Satoh, M., Sakamoto, S., Koga, S., and Nawata, H. (1990) *Clin. Chim. Acta 189*, 303−311.
14. Steiner, D. F., Quinn, P. S., Chan, S. J., Marsh, J., and Tager, H. S. (1980) *Ann. N.Y. Acad. Sci. 343*, 1−16.
15. Roghani, A., and Zannis, V. I. (1988) *Biochemistry 27*, 7428−7435.
16. Stoffel, W., and Niedel, B. (1985) *Biol. Chem. Hoppe-Seyler 366*, 181−187.
17. Hakes, D. J., and Dixon, J. E. (1992) *Anal. Biochem. 202*, 293−298.
18. Pyle, L. E., Sawyer, W. H., Fujiwara, Y., Mitchell, A., and Fidge, N. H. (1996) *Biochemistry 35*, 12046−12052.
19. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−256.
20. Allan, C. M., Tetaz, T., and Fidge, N. H. (1991) *J. Lipid Res. 32*, 595−601.
21. Sviridov, D., Pyle, L., and Fidge, N. (1996) *Biochemistry 35*, 189−196.
22. Sparrow, D. A., Lee, B. R., Laplaud, P. M., Auboiron, S., Bauchart, D., Chapman, M. J., Gotto, A. M., Jr., Yang, C. Y., and Sparrow, J. T. (1992) *Biochim. Biophys. Acta 1123*, 145−150.
23. Yang, C. Y., Gu, Z. W., Patsch, W., Weng, S. A., Kim, T. W., and Chan, L. (1987) *FEBS Lett. 224*, 261−266.
24. Chung, H., Randolph, A., Reardon, I., and Heinrikson, R. L. (1982) *J. Biol. Chem. 257*, 2961−2967.
25. Polites, H. G., Melchior, G. W., Castle, C. K., and Marotti, K. R. (1986) *Gene 49*, 103−110.
26. Yang, C. Y., Yang, T., Pownall, H. J., and Gotto, A. M., Jr. (1986) *Eur. J. Biochem. 160*, 427−431.
27. Poncin, J. E., Martial, J. A., and Gielen, J. E. (1984) *Eur. J. Biochem. 140*, 493−498.

BI002025G