Apolipoprotein J Is Associated with Paraoxonase in Human Plasma†

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Received April 6, 1993; Revised Manuscript Received September 29, 1993•

ABSTRACT: Apolipoprotein J (apoJ)-containing high-density lipoproteins (HDL), isolated from human plasma by immunoaffinity chromatography, are associated with apoAI and a protein of approximately 44 kDa. In order to advance our understanding of apoJ's role in the vasculature, a comprehensive investigation was performed to identify and characterize this 44-kDa protein and to study its interaction with apoJ. The 44-kDa protein, a monomeric glycoyslated polypeptide, was identified by N-terminal sequencing as serum paraoxonase. Paraoxonase exists in two oxidation states: one contains all free cysteines while the other has one disulfide bond between Cys_{42} and Cys_{284}. Northern analysis of eight human tissues shows paraoxonase message present only in the liver. The majority of apoJ/paraoxonase-HDL are 90–140 kDa; however, not all of the plasma paraoxonase is associated with apoJ. The specificity of the apoJ/paraoxonase interaction, inferred by the constant mole ratio of the two proteins in affinity-purified apoJ-HDL, is confirmed in direct binding assays. For purified proteins, there is more than a 5-fold increase in the apparent affinity of apoJ for immobilized paraoxonase as the paraoxonase coating concentration is increased from 0.5 to 2.0 μg/mL. Both oxidation states of paraoxonase bind to apoJ with equal affinity. Our data combined with other evidence suggest that the plasma link of apoJ with paraoxonase will be implicated as a predictor of vascular damage.

The newest member of the group of high-density lipoprotein (HDL)[1] apolipoproteins, apoJ, has attracted significant scientific interest since it was initially purified from ram rete testis fluid (Blaschuk et al., 1983). Identified as an apolipoprotein in 1990 (de Silva et al., 1990b), apoJ was shown to be associated with discrete populations of HDL with α2 electrophoretic mobility (Stuart et al., 1992) that also contain apoAI and cholesteryl ester transfer activity (de Silva et al., 1990b). ApoJ message is expressed in nearly all mammalian tissues examined (Collard & Griswold, 1987; de Silva et al., 1990a), and apoJ protein is present in most body fluids (de Silva et al., 1990a; Sylvester et al., 1991). ApoJ has been implicated in diverse biological processes, including lipid transport (de Silva et al., 1990b; James et al., 1991; Jenne et al., 1991), sperm maturation (Collard & Griswold, 1987), complement inhibition (Choi et al., 1989), programmed cell death (Buttyan et al., 1989), tissue remodeling (Danik et al., 1991), membrane recycling (Palmer & Christie, 1990), and epithelial barrier cytoprotection (Jordan-Starck et al., 1992). Most recently, apoJ has been proposed to play a role in Alzheimers disease, possibly acting as a "solubility chaperone" to the β-amyloid peptide, which has been implicated in plaque formation (Wisniewski et al., 1993).

Speculation regarding apoJ's function has been based on such parameters as its interaction with certain classes of molecules *in vivo*, its biochemical properties shown by *in vitro* assays, its constitutive pattern of tissue expression, and its marked induction in response to a variety of pathologic stimuli (Jenne & Tschopp, 1992; Jordan-Starck et al., 1992). For example, apoJ's role in regulation of the complement cascade is indicated by its association with terminal complement complex proteins *in vivo* as well as its ability to inhibit complement-mediated cell lysis *in vitro* (Choi et al., 1989; Murphy et al., 1988). In plasma, apoJ's role in lipid transport is supported by its association with lipids and apoAI, the major protein component of HDL (de Silva et al., 1990b). These associations, confirmed by other investigators (James et al., 1991; Jenne et al., 1991), strongly suggest that apoJ functions in extracellular lipid transport.

Our goal to understand the physiological function(s) of the plasma form of apoJ led us to investigate a unique apoJ-associated protein of approximately 44 kDa. We consistently observed a protein of 44 kDa associated with immunoaffinity-isolated apoJ, and subsequently identified this protein as serum paraoxonase. This investigation was carried out to answer several basic questions regarding apoJ's capacity for direct interaction with paraoxonase, as well as to determine the reason for paraoxonase's observed structural heterogeneity. During this investigation, Blatter et al. (1993) published the identification of a distinct HDL subspecies defined by apoJ and serum paraoxonase, verifying our identification and association of this protein with apoJ. A definition of the association of

† This research was supported by NIH Program of Excellence Grant HL41496 (J.A.K.H.) and by National Institutes of Health Grant ES05194 (C.E.F.). G.J.K. was the recipient of support from NIH Training Grant HL07382. T.C.J.-S. was supported by NIH Program of Excellence Grant HL41496.

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• Abstract published in *Advance ACS Abstracts*, January 15, 1994.

[1] Abbreviations: ABD-F, 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole; apo, apolipoprotein; BME, β-mercaptoethanol; BSA, bovine serum albumin; CBB, Coomassie brilliant blue; DAB, 3,3′-diaminobenzidine; HDL, high-density lipoprotein(s); HRP, horseradish peroxidase; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PDB, plasma density buffer; PMSF, phenylmethanesulfonyl fluoride; PNPP, *p*-nitrophenyl phosphate; PVDF, poly(vinylidene difluoride); SDS, sodium dodecyl sulfate; SSC, standard saline citrate; SSPE, standard saline K_2-EDTA; TBP, tributylphosphine; TFA, trifluoroacetic acid; TFMS, trifluoromethanesulfonate; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone.

0006-2960/94/0433-832$04.50/0

apoJ with paraoxonase may provide insight into (1) the regulation of apoJ function and/or (2) the metabolic pathway of apoJ within the plasma compartment.

EXPERIMENTAL PROCEDURES

Materials. 4-(Aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) was obtained from Wako Chemicals. Tributylphosphine (TBP) and dimethyl sulfoxide were purchased from Aldrich. HPLC-grade acetonitrile was obtained from Fisher Chemical Co. C_{18} semipreparative and analytical HPLC columns and the C_4 analytical HPLC column were obtained from Vydac Separations Group. Trifluoroacetic acid (TFA) was obtained from Pierce. PNGase-F was obtained from Genzyme. Polyacrylamide gel reagents, poly(vinylidene difluoride) (PVDF) membrane, and horseradish peroxidase (HRP)-conjugated secondary antibodies for electroimmunoblotting were obtained from BioRad Laboratories. Nitrocellulose paper was purchased from Hoefer. Ampholines and 4–30% native gradient gels were obtained from Pharmacia LKB Biotechnology Inc.; 11–23% denaturing gradient gels (PhorCast) and ^{125}I-conjugated secondary antibodies were obtained from Amersham. Molecular weight standards were obtained from Gibco–Bethesda Research Laboratories. Ribi adjuvant was purchased from Ribi Immunochem Research Inc. Multiple tissue Northern blot was purchased from Clontech Laboratories. Other chemicals and reagents were purchased from Sigma or Fisher. ApoE was purified from human plasma very low density lipoproteins by the method of Cardin et al. (1984).

Immunoaffinity Purification of ApoJ. ApoJ was isolated from human plasma by immunoaffinity chromatography as previously described (de Silva et al., 1990c). Briefly, 250–500 mL of plasma diluted 1:3 in 10 mM NaH_2PO_4, 150 mM NaCl, 3 mM KCl, and 3 mM KH_2PO_4, pH 7.4 (PBS), containing protease inhibitors [1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM EDTA, 1 mM benzamidine, 25 Kallikrein units (KU)/mL aprotinin, and 0.01% NaN_3] was chromatographed at 4 °C on a mAb11-Affigel column (de Silva et al., 1990c). The column was washed extensively with 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM EDTA (PDB) to remove unbound protein, and bound protein was eluted in 100 mL of 1 M acetic acid (HOAc). The eluted protein (termed mAb11 eluate) was dialyzed immediately against 10 mM NH_4HCO_3 and concentrated in a Savant Speed-Vac. Immunoaffinity-purified apoJ was applied to a C_{18} semipreparative HPLC column equilibrated with buffer A (0.1% TFA/H_2O). Protein was resolved with an increasing gradient of buffer B (0.1% TFA/CH_3CN) at a flow rate of 4.7 mL/min. The gradient used to separate proteins consisted of 0% buffer B over 0–4 min, 0–35% buffer B over 4–8 min, 35–48% buffer B over 8–15 min, and 48–70% buffer B over 15–60 min.

Purification of Serum Paraoxonase. mAb11 eluate, dialyzed against 10 mM NH_4HCO_3 as previously described, was lyophilized. The dried protein complex was solubilized in 2 mL of 10 mM Tris-HCl, pH 6.8, 10% glycerol, and 2% SDS, evenly applied to two 10% SDS gels (16 × 18 cm), and electrophoresed at 30 mA for 4–6 h. The protein complex was electrophoresed without reductant for isolation of the paraoxonase A and paraoxonase B species, or with reductant (20 mM DTT) for isolation of both species. After electrophoresis, bands of interest were visualized by brief exposure to 1 M KCl, and then excised from the gel. The protein was then electroeluted overnight at 100 V at room temperature in 40 mM Tris–borate, pH 8.4, and 0.5 mM EDTA using the Schleicher & Schuell Elutrap device. After electroelution, paraoxonase was either dialyzed against an appropriate buffer or precipitated from the electroelution buffer by the addition of 4 volumes of cold acetone and storage at −20 °C for 2–8 h. After precipitation, the protein was pelleted by centrifugation for 5 min at 2800*g* and partially dried under a N_2 stream prior to solubilization in an appropriate buffer. Complete drying of the pellet at this stage rendered the paraoxonase insoluble.

Alternatively, paraoxonase was obtained during the immunoaffinity purification of apoJ as previously described. In the HPLC separation step, paraoxonase eluted just following the apoJ peak. The paraoxonase peak was collected, rechromatographed, and either acetone-precipitated or dialyzed against an appropriate buffer.

Two-Dimensional Gel Electrophoresis. The first dimension consisted of isoelectric focusing (Anderson & Anderson, 1977; O'Farrell, 1975); the second, SDS–PAGE. Isoelectric focusing gels were cast in glass tubes (3 mm × 11 cm) with a pH 4–8 gradient. Protein samples were solubilized in 9.5 M urea, 5% BME, 2% SDS, 10% glycerol, 5% ampholines, and 3% Nonidet P-40, and incubated for 30 min at 37 °C. Electrophoresis was performed at 400 V for 12 h, and the gels were carefully extruded and layered over a 10% SDS gel. The subsequent electrophoresis was performed at 35 mA for 5 h, and the acrylamide gels were stained for protein with 40% methanol, 10% acetic acid, and 1% Coomassie brilliant blue (CBB).

Deglycosylation. Proteins (100 μg) were deglycosylated in 20 mM Na_2HPO_4, pH 7.3, 10 mM EDTA, and 0.6% octyl β-glucoside. PNGase-F was added at 12 units/mL and allowed to react for 24 h at 37 °C. The deglycosylated samples were dialyzed against 10 mM NH_4HCO_3 and lyophilized.

Amino Acid Analysis and Protein Sequencing. Proteins, electroblotted onto a PVDF membrane, were sequenced on a Porton Instruments Model 2090E gas-phase sequencer, and the phenylthiohydantoin amino acids were analyzed with the on-line microbore HPLC. Amino acid analysis was determined with the Waters PICO-TAG system. All amino acid analyses and protein sequencing were performed by the Protein Chemistry Core Facility in the Department of Pharmacology and Cell Biophysics, University of Cincinnati.

Paraoxonase Assay. Paraoxonase activity was determined by a previously described assay (pH 8.5) that is selective and optimized for the polymorphic paraoxonase/arylesterase (Furlong et al., 1989). The paraoxonase assay contained 700–795 μL of assay buffer, 5–100 μL of sample, and 200 μL of 6 mM paraoxon substrate solution. The assay was initiated by the addition of the substrate solution, and the paraoxonase activity was measured spectrophotometrically by following the formation of *p*-nitrophenol at 405 nm.

Preparation of Anti-Paraoxonase Antibodies. Polyclonal antibodies against electroeluted and HPLC-purified paraoxonase were raised in New Zealand White female rabbits (>1.5 kg total body weight). Antigen purity was assessed by SDS–PAGE. Rabbit preimmune serum displayed no reactivity to human plasma, based on electroimmunoblot analysis. On day 1, 50 μg of purified paraoxonase was suspended in 1 mL of Ribbi adjuvant and administered subcutaneously in three injections (200 μL each) and intramuscularly in each hip (200 μL each). The animals were boosted after 2, 5, and 8 weeks with identical injections. Antiserum was collected after 1 week postinjection and 1 week after each booster. Antibody titering by electroimmunoblot analysis of human plasma was performed on each antiserum. Slight reactivity against apoJ

was detected in the electroimmunoblot analysis of plasma, probably due to apoJ contamination acquired during the HPLC purification of paraoxonase. Therefore, the antiserum was incubated with pure paraoxonase electroblotted onto nitrocellulose to obtain monospecific antibodies (Smith & Fischer, 1984). After the nitrocellulose was washed, bound antibodies were eluted with three washes of 5 mM glycine, pH 2.3, 0.5 M NaCl, 0.5% Tween 20, and 100 μg/mL BSA and immediately neutralized. The eluted antibodies were used in all subsequent electroimmunoblot analyses.

Electroblot Procedure. Protein samples were solubilized in 10 mM Tris-HCl, pH 6.8, 10% glycerol, and 2% SDS, with and without 1% β-mercaptoethanol (BME), and separated on 10% SDS gels. The acrylamide gels were then stained for protein (40% methanol, 10% acetic acid, and 1% CBB) or transferred to nitrocellulose or a PVDF membrane at 250 mA for 2 h in 25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 20% methanol.

Immunostaining Procedure. The electroblots for immunostaining were incubated at 4 °C overnight with primary antibody in blocking buffer without Tween 20. After a thorough washing, the blots were incubated for 2 h at room temperature with HRP-conjugated secondary antibody (1:3000 dilution in blocking buffer without Tween 20). Specific reactivity was determined by developing the blots in a solution containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mg/mL 3,3′-diaminobenzidine (DAB), 5 mM NiCl, and 0.3% H_2O_2.

Hybridization of Paraoxonase cDNA with RNA. A multiple-tissue Northern blot of poly(A^+) RNA from eight different human tissues (purchased from Clontech Laboratories) was probed with a full-length human paraoxonase cDNA (Hassett et al., 1991). Each lane contained 2 μg of poly(A^+) RNA from either human heart, brain, placenta, lung, liver, skeletal muscle, kidney, or pancreas. Prior to hybridization, the blot was incubated for 4 h at 42 °C in 5× SSPE (1× SSPE: 0.15 M NaCl, 0.01 M NaH_2PO_4, and 1 mM Na_2EDTA, pH 7.4), 10× Denhardt's solution, 100 μg/mL sheared salmon sperm DNA, 50% freshly deionized formamide, and 2% SDS. Hybridization with the radiolabeled human paraoxonase cDNA was performed at 42 °C for 24 h in the solution described above. The blot was washed 3× for 40 min at room temperature in 2× SSC (1× SSC: 0.15 M NaCl/0.015 M sodium citrate, pH 7.0)/0.05% SDS, and then 2× at 50 °C in 0.1× SSC/0.1% SDS for 40 min. The blot was exposed to Kodak X-OMAT AR film for 3 days at −70 °C using two intensifying screens.

ABD-F Labeling. Protein samples or tryptic peptides (various concentrations) were brought to a volume of 0.7 mL with H_2O, and 0.1 mL of a freshly mixed solution containing 1.0 M $NaBO_3$, pH 8.0, 8 mM EDTA, and either 2 mM ABD-F (for alkylation of free cysteines) or 2 mM ABD-F + 2 mM TBP (for alkylation of all cysteines) was added (Kelso et al., 1991). The samples were vortexted and allowed to react for 10 min at 60 °C. Quantitation of cysteine content was performed on a Beckman DU-6 spectrophotometer, monitoring 385 nm and using an extinction coefficient of 7800 M^{-1} cm^{-1} for ABD-F-labeled cysteine. The relative fluorescence of treated peptides was determined manually, using a Perkin-Elmer 650-10S spectrofluorometer, or with a Shimadzu RF-535 HPLC fluorescence detector (excitation at 385 nm, emission at 520 nm).

ApoJ-HDL Particle Analysis. Two-dimensional electrophoretic analysis (Castro & Fielding, 1988) of the apoJ-HDL in plasma was performed, consisting of agarose electrophoresis followed by nondenaturing gradient gel electrophoresis. Plasma samples were obtained from healthy fasted donors and protease inhibitors added. Plasma (0.5 mL) was loaded into every other lane of a 1% agarose gel (Ciba) and electrophoresed in 50 mM barbital buffer, pH 8.6, containing 1 mM EDTA at 10 °C in a Bio-Rad flatbed electrophoresis apparatus at 125 V for approximately 1 h. Each lane of the agarose gel was excised and annealed to a nondenaturing 4–30% polyacrylamide gel with 0.75% agarose in Tris–borate buffer. The gradient gel was electrophoresed in Tris–borate buffer, pH 8.3, at 4 °C for 2040 V-h. Equilibrium was not achieved to retain small (<70 kDa) particles. Gels were soaked for 2 h in transfer buffer containing 0.5% SDS, rinsed in transfer buffer without SDS for 5 min, and electroblotted at 200 mA onto two sheets of a nylon membrane (Zetaprobe, BioRad) for 54 h at 4 °C. A strip of PVDF membrane was placed between the lane containing the standards and the nylon membrane, the standards were stained with Ponceau S, and a standard curve was constructed to estimate molecular weights. The membranes were then incubated simultaneously with the rabbit anti-paraoxonase polyclonal antibody and the mouse anti-apoJ mAbII antibody, diluted 1:5000 in blocking buffer as previously described. Paraoxonase was visualized with goat anti-rabbit–HRP conjugate, diluted 1:5000. ApoJ was visualized by incubation with 1.5 μCi of sheep anti-mouse ^{125}I-IgG and exposed to Kodak X-OMAT AR film at −70 °C for 3 days.

ApoJ–Paraoxonase Association. Freshly drawn human plasma was diluted 1:3 in PBS containing protease inhibitors. ApoJ was depleted from plasma by repeated extractions with an apoJ immunoaffinity column (Stuart et al., 1992). Aliquots of HPLC-purified apoJ were dried in a Savant vacuum concentrator and resolubilized in 26.5 mL of apoJ-depleted plasma, incubated for 5 h at 37 °C with gentle agitation, and applied to apoJ immunoaffinity column (1 × 5 cm). Columns were washed with 500 mL of 10 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA, pH 7.4 (PDB), and 100 mL of PDB with 500 mM NaCl before elution with 40 mL of 1 M acetic acid. Eluates were dialyzed into 10 mM NH_4HCO_3 containing 1 mM Na_2EDTA and then into H_2O containing 0.02% NaN_3. Protein concentrations were determined by the Bradford method (BioRad). Equivalent volumes of each eluate were vacuum-concentrated, electrophoresed under denaturing conditions on 11–23% PhorCast gels, and stained with 40% methanol, 10% acetic acid, and 1% CBB. Relative amounts of apoJ and paraoxonase were determined by laser densitometric scanning (LKB Ultrascan). Standard curves, made to correct for disproportionate staining of the proteins, were generated with known amounts (amino acid analysis) of apoJ and paraoxonase in the same gel system.

ApoJ–Paraoxonase Binding Assay. Paraoxonase was prepared by electroelution from SDS gels as previously described. The paraoxonase was solubilized in 50 mM sodium acetate, pH 5.1, containing 0.02% NaN_3, and protein concentrations were determined by the Bradford method (Bio-Rad). Paraoxonase was adsorbed to the surface of 96-well polystyrene microtiter plates (Nunc-Immuno plate I) by incubation for 2 h at 37 °C. The wells were blocked overnight at 4 °C with a buffer consisting of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5% nonfat dry milk, 0.01% antifoam A, and 0.05% Tween 20 (blocking buffer). ApoJ in blocking buffer was added to the wells and incubated for 2 h at 37 °C. The wells were washed 5× with blocking buffer, and anti-apoJ primary antibody (1:500 dilution of mAb11 in blocking buffer) was added for 2 h at 37 °C. The wells were washed 5× with

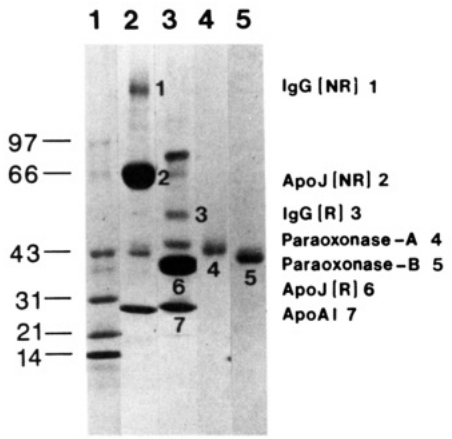

FIGURE 1: A 44-kDa protein is present in apoJ-HDL. Freshly drawn human plasma, diluted 1:3 in phosphate-buffered saline containing protease inhibitors, was chromatographed on an mAb11-AffiGel column as described previously (de Silva et al., 1990b), and apoJ-HDL was eluted and analyzed by SDS–PAGE. Proteins were stained with CBB. Lane 1, BRL molecular mass standards; lanes 2 and 3, mAb11 eluate (30 μg) in the absence and presence of 1.0% BME, respectively; lanes 4 and 5, electroeluted paraoxonase-A (5 μg) and paraoxonase-B (5 μg), respectively.

blocking buffer before HRP-conjugated secondary antibody (1:200 dilution in blocking buffer) was added for 1 h at 37 °C. Finally, the wells were washed 3× with blocking buffer and then 2× with PDB + 2 mM Ca^{2+}. The plates were developed for 25 min at room temperature with a substrate solution consisting of 1 mg/mL *p*-nitrophenyl phosphate (PNPP), 10 mM diethanolamine (pH 9.5), and 0.5 mM $MgCl_2$. The reaction was stopped by addition of 50 μL of 3 M NaOH. Product formation was monitored by measuring the absorbance at 405 nm, using a Thermomax plate reader (Molecular Devices).

RESULTS

Isolation of a 44-kDa ApoJ-Associated Protein from Human Plasma. The presence of a 44-kDa plasma protein in eluates from anti-apoJ affinity columns was first noted by de Silva et al. (1990b). SDS–PAGE analysis of a typical eluate under nonreducing conditions, shown in Figure 1 (lane 2), revealed four major species of 160, 67, 44, and 28 kDa. ApoJ corresponded to the 67-kDa species. Under reducing conditions (lane 3), apoJ was predominantly the broad 35-kDa species; a fraction of apoJ maintained resistance to reduction even under harsh reducing conditions, and migrated at 70 kDa. IgG, identified by amino-terminal sequence analysis, corresponded to the 160-kDa species under nonreducing conditions (lane 2) and the 55-kDa species under reducing conditions (lane 3). The 28-kDa species (lanes 2 and 3) was identified as apoAI by electroimmunoblott and amino-terminal sequence analysis (de Silva et al., 1990b), while the identity of the 44-kDa species was unknown. Without reduction, the 44-kDa protein appeared as a doublet, designated 44-A (upper band in lane 2) and 44-B (lower band in lane 2). Upon reduction, the doublet disappeared, and a single 44-kDA species appeared (lane 3). Both 44-A and 44-B were excised from preparative SDS–PAGE gels and electroeluted. Results of SDS–PAGE analysis of the purified proteins under nonreducing conditions are shown in Figure 1, where 44-A appears in lane 4 and 44-B appears in lane 5. Both forms were quite stable, with no change detected by SDS–PAGE analysis after storage for 3 months in electroelution buffer (data not shown). When electroeluted 44-A and 44-B were subjected to enzymatic deglycosylation with PNGase-F, which specifically cleaves N-linked carbohydrate (Plummer et al., 1984), the apparent molecular weight of each form decreased by approximately 20%; the differential migration pattern of the A and B forms was maintained (data not shown). Two-dimensional gel electrophoresis of a mixture of native 44-A plus 44-B yielded a single band with a p*I* of approximately 5.2 (data not shown).

44-A and 44-B Have Identical N-Terminal and Tryptic Peptide Sequences. When electroeluted 44-A and 44-B were electroblotted to a PVDF membrane and subjected to protein sequence analysis separately, they yielded identical N-terminal sequences (AKLIALTLLGMGLALFRNHQ). Additionally, internal tryptic peptides (eluting at 44.3 min in 42% acetonitrile) isolated from both forms had identical protein sequences (YVYIAELLAHK).

The 44-kDa Protein Is Human Serum Paraoxonase. Using the N-terminal sequence, a search of the NIH Genbank revealed a 100% homology to human serum paraoxonase (Hassett et al., 1991). Likewise, all of the internal tryptic peptide sequences obtained were found within the published sequence of serum paraoxonase. For example, the internal peptide described above corresponded to residues 234–245 of paraoxonase. Mature paraoxonase is a 355 amino acid protein with a calculated molecular mass of 39.7 kDa and an estimated p*I* of 4.9. Four potential N-glycosylation sites are found in the paraoxonase sequence, based upon the N-X-(S/T) consensus sequence (Marshall, 1974). No paraoxonase activity was detected in either paraoxonase-A (4.4 μg) or paraoxonase-B (21.4 μg) isolated from preparative SDS–PAGE gels, or in eluates from anti-apoJ affinity columns (8.2 μg), while control purified human paraoxonase (2.5 μg) (Furlong et al., 1991) displayed a paraoxonase activity of 0.240 ΔOD/min. The absence of catalytic activity was not unexpected for the electroeluted paraoxonase samples, since Furlong et al. (1991) have reported only occasional success in renaturing active human paraoxonase following SDS gel electrophoresis. The reason for the lack of activity in eluates from an anti-apoJ affinity column is not known, but could be a consequence of the acidic environment encountered by paraoxonase during the column elution step. ApoJ had no effect on the catalytic activity of paraoxonase when equal amounts of active human paraoxonase (2.5 μg) and HPLC-purified apoJ (2.5 μg) were mixed and allowed to react overnight.

Human Paraoxonase cDNA Hybridizes to a 1.5-kb Message in Liver. Since apoJ message is produced in numerous human tissues (de Silva et al., 1990a; Aronow et al., 1993), a Northern blot analysis with a full-length human paraoxonase cDNA was performed to determine if paraoxonase displays a pattern of expression similar to that of apoJ. Hassett et al. (1991) had performed a Northern blot analysis with RNA isolated from four rabbit organs and reported the presence of paraoxonase-specific RNA in liver only. Similarly, in our Northern blot analysis of eight human tissues, high levels of message were detected in human liver (Figure 2), while poly-(A+) RNA isolated from human heart, brain, placenta, lung, skeletal muscle, kidney, or pancreas failed to hybridize to the paraoxonase cDNA (Figure 2). The paraoxonase cDNA hybridized to a single liver mRNA of approximately 1.5 kb.

The Two Forms of Paraoxonase Differ by a Disulfide Bond. In an attempt to determine the difference between the two forms of paraoxonase evident by SDS–PAGE analysis (Figure 1), the oxidation state of the cysteine residues was investigated. Utilizing the sulfhydryl-specific reagent ABD-F, the oxidation state of the three cysteine residues was determined. A comparison of the ABD-alkylated paraoxonase-A and paraox-

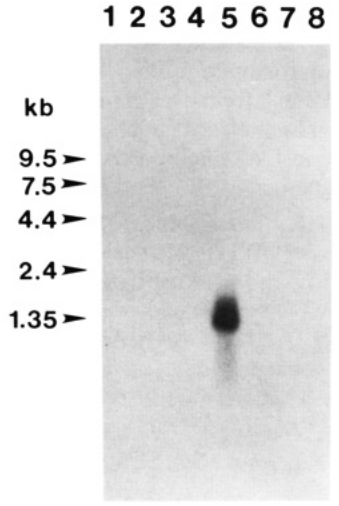

FIGURE 2: Northern blot analysis of the distribution of paraoxonase mRNA in human tissues. Poly(A+) RNA (2 μg) from eight different human tissues was probed with a full-length human paraoxonase cDNA, as described under Experimental Procedures. Lanes 1–8, poly(A+) RNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas, respectively. RNA size markers are indicated at the left.

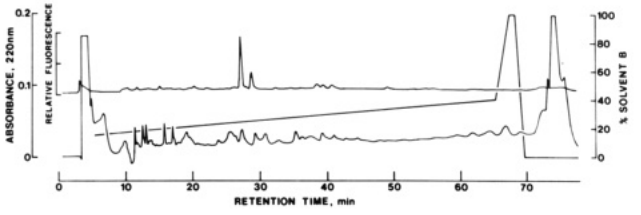

FIGURE 3: HPLC separation of trypsin-digested paraoxonase-B. ABD-F-labeled paraoxonase-B was digested with trypsin and applied to a Vydac C_{18} reverse-phase column equilibrated with 0.1% TFA/H_2O. The peptides were separated with an increasing gradient of 0.1% TFA/acetonitrile. Upper trace, relative fluorescence; lower trace, 220 nm, 0.2 AUFS.

onase-B without reduction indicated a 3:1 ratio of free sulfhydryls in paraoxonase-A compared to that of paraoxonase-B (data not shown). Since the primary sequence contains only three cysteines (Hassett et al., 1991), these results are consistent with the presence of one disulfide bond in paraoxonase-B and none in paraoxonase-A.

Paraoxonase-B Forms a Disulfide Bond between Cys_{42} and Cys_{284}. When the sample of ABD-alkylated paraoxonase-B without reduction was digested with trypsin and separated by HPLC, one major peak and minor fluorescent peak were observed, corresponding to ABD-Cys-containing peptides (Figure 3). The major fluorescent peak eluted at 25.6 min in 24.8% acetonitrile, and the minor fluorescent peak eluted at 29.1 min in 26.3% acetonitrile. These peaks were collected, rechromatographed, and submitted for sequence analysis. The sequence of peak 1 determined through 15 cycles (IFFYDSENPPASEVL) corresponded to position 291–305 in the mature paraoxonase sequence. The sequence of peak 2 determined through 6 cycles (ALYCEL) corresponded to positions 350–355 in the primary sequence. The presence of ABD-Cys in peak 1 was evident in both the HPLC fluorescent trace of the purified peptide and the amino acid analysis of the peptide prior to sequencing, and would correspond to Cys_{353}. Therefore, since Cys_{353} was determined to be the free cysteine, the disulfide bond forms between Cys_{42} and Cys_{284}.

Both Forms of Paraoxonase Are Present in Plasma. A polyclonal antibody was raised against both electroeluted and HPLC-purified paraoxonase, and used to screen plasma for

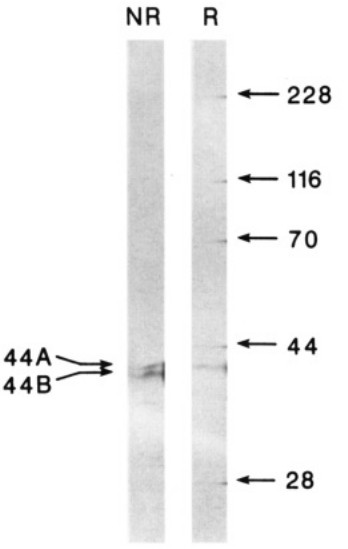

FIGURE 4: Both 44-A and 44-B forms of paraoxonase are present in plasma. Human plasma (50 μg) was loaded on a SDS–PAGE gel in the absence (nonreduced, NR) or presence (reduced, R) of BME, and electrophoresed at 20 mA for 1.5 h. The proteins were transferred to nitrocellulose for immunostaining with anti-paraoxonase.

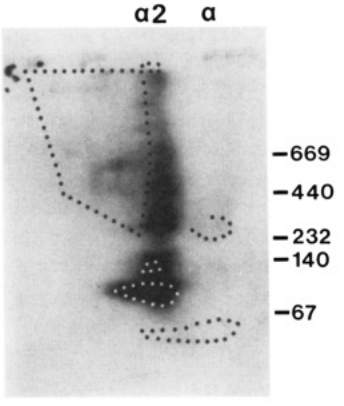

FIGURE 5: A subpopulation of apoJ-HDL contains paraoxonase. Human plasma (4 μl) was subjected to 2D electrophoretic analysis, as outlined under Experimental Procedures. ApoJ and paraoxonase were visualized simultaneously by immunostaining, using mAbII followed by sheep anti-mouse ^{125}I-IgG and anti-paraoxonase followed by goat anti-rabbit IgG–HRP. An autoradiograph of the apoJ distribution pattern is depicted with dotted lines added to indicate the area of paraoxonase staining. White dots indicate the overlap of paraoxonase and apoJ.

the presence of the two forms of the protein. Both forms were present in plasma, based on electroimmunoblot analysis (Figure 4). The A:B ratio varied between individuals. There was no noticeable change in immunoreactivity with the anti-paraoxonase antibody when normal plasma was compared to apoJ-depleted plasma (data not shown), indicating that apoJ is associated with a small fraction of the total paraoxonase present in plasma.

A Subpopulation of ApoJ-HDL Contains Paraoxonase. The distribution of paraoxonase in apoJ-HDL was investigated by 2D electrophoresis. Freshly drawn plasma was subjected to 2D electrophoretic analysis. The majority of apoJ migrated in the α2 position (darkened areas in Figure 5), where the apparent molecular masses of the apoJ species concentrated in the 70–140-kDa range with other species at 232 kDa and higher. Paraoxonase was detected over a wide area, spanning both the α and β positions (dotted areas in Figure 5), with the most intense immunostaining occurring in the α-migrating HDL region. The paraoxonase profile has been traced onto an autoradiograph depicting apoJ distribution to aid in

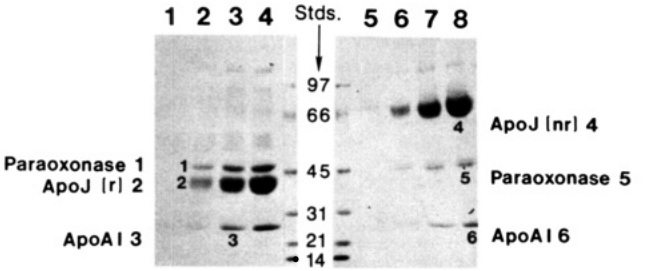

FIGURE 6: Paraoxonase interacts with apoJ. Purified apoJ was added to 26.5 mL of apoJ-depleted plasma, and apoJ complexes were recovered by chromatography on mAbII-AffiGel as outlined under Experimental Procedures. Complexed proteins were separated by SDS–PAGE after reduction by BME (lanes 1–4) or without reduction (lanes 5–8), and quantitated as the peak area by densitometric scanning of the CBB-stained gel. Lanes 1–4 and 5–8 represent 0, 160, 310, or 470 μg of purified apoJ added to apoJ-depleted plasma.

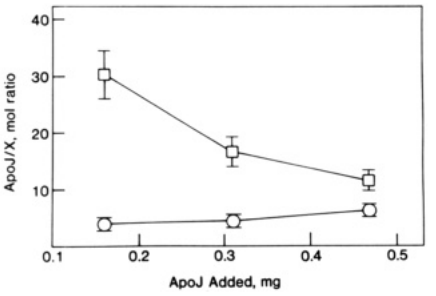

FIGURE 7: Ratio of apoJ/paraoxonase in recovered apoJ complexes remains constant throughout the range of purified apoJ added back to apoJ-depleted plasma. The apoJ/apoAI (□) and apoJ/paraoxonase (○) mole ratios were calculated as described under Experimental Procedures. The means of triplicate measurements ± standard errors are displayed.

evaluating the apoJ–paraoxonase relationship (Figure 5). The overlap of apoJ and paraoxonase occurred primarily in the 90–140-kDa range. However, the majority of apoJ did not appear to comigrate with paraoxonase.

Paraoxonase Specifically Associates with ApoJ. The interaction between paraoxonase and apoJ was investigated, using the method previously employed for examining apoJ/apoAI interactions (Stuart et al., 1992). HPLC-purified apoJ was added to apoJ-depleted plasma in increasing amounts, and apoJ was extracted from the plasma by immunoaffinity chromatography after each addition. Paraoxonase and apoAI were removed along with apoJ in each extraction (Figure 6). The amount of apoAI removed was proportional to the amount of apoJ added in each case (R = 0.90), and displayed a decreasing apoJ/apoAI mole ratio (Figure 7). The amount of paraoxonase removed was also proportional to the amount of apoJ added in each case (R = 0.98), and displayed a near constant apoJ/paraoxonase mole ratio of 4.7 ± 0.91 (Figure 7). This value was in reasonable agreement with the ratio of 8.2 ± 2.1 obtained when apoJ was extracted by immunoaffinity chromatography from control plasma (stored at 4 °C for 1 week).

To determine whether purified paraoxonase and apoJ exhibit affinity for each other, a direct binding assay was developed. Wells of microtiter plates to which paraoxonase had been adsorbed were incubated with increasing concentrations of apoJ, and binding was detected using a monoclonal antibody directed against apoJ (Figure 8). When increasing concentrations of paraoxonase were incubated with apoE, no binding was detected by using a polyclonal antibody directed against apoE (Lilly-Stauderman et al., 1993) (Figure 9). Since there was no difference in the apparent affinity of apoJ for

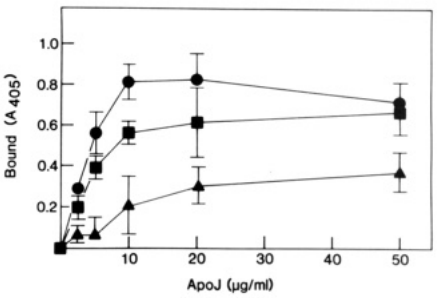

FIGURE 8: Binding of purified apoJ to purified paraoxonase. The absorbance associated with apoJ bound to immobilized human paraoxonase [coating concentrations of 0.5 (▲), 1.0 (■), or 2.0 μg/mL (●)] was measured as indicated under Experimental Procedures. The means of triplicate measurements ± standard errors are displayed.

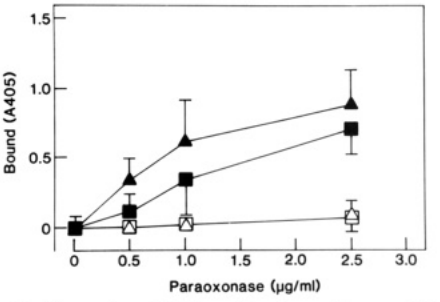

FIGURE 9: Binding of purified apoJ and apoE to purified paraoxonase-A and paraoxonase-B. The absorbance associated with 10 μg/mL apoJ bound to immobilized paraoxonase-A (■) or paraoxonase-B (▲) and 5 μg/mL apoE bound to immobilized paraoxonase-A (□) or paraoxonase-B (△) was measured as indicated under Experimental Procedures. The means of triplicate measurements ± standard errors are displayed.

Table 1: Effect of Increasing Paraoxonase Concentration on the Apparent Affinity of ApoJ for Paraoxonase[a]

[paraoxonase] (μg/mL)	$K_{D(app)}$ (μg/mL)
0.5	16.3 ± 5.6
1.0	4.6 ± 0.8
2.0	2.7 ± 1.2

[a] The binding of apoJ to paraoxonase immobilized on microtiter plates was determined as described under Experimental Procedures. The values shown were obtained by fitting the equation describing saturation binding to the data shown in Figure 8.

paraoxonase-A vs paraoxonase-B (Figure 9), the binding assays were performed with paraoxonase-B. Although saturable binding of apoJ to immobilized paraoxonase occurred at the concentrations examined, a linear transformation of the saturation binding data indicated complex binding phenomena. When the binding data from Figure 8 were fit to the equation describing saturation binding, $B = B_{max}/[1 + (K_D/[\text{Free}])]$, a greater than 5-fold increase in the apparent affinity of apoJ for immobilized paraoxonase was found as the coating concentration of paraoxonase was increased from 0.5 to 2.0 μg/mL (Table 1).

DISCUSSION

In this study, we have characterized yet another novel interaction attributable to the multifunctional, widely expressed protein known as apoJ. We have determined that apoJ specifically associates with paraoxonase in human plasma. Our results clearly demonstrate a specific apoJ/paraoxonase interaction both *in vitro* and *in vivo*. Affinity-purified apoJ-HDL, isolated from multiple normolipemic donors, are associated with a constant amount of paraoxonase, and when apoJ is reintroduced into apoJ-depleted plasma, paraoxonase

is removed with apoJ by an anti-apoJ affinity column in a constant apoJ/paraoxonase mole ratio. Specific assays of purified paraoxonase binding to apoJ verified this interaction. This work extends the studies by Blatter et al. (1993), who identified an apoJ/paraoxonase HDL species, by documenting the specificity of the interaction and establishing direct apoJ–paraoxonase binding. In addition, the structural heterogeneity of serum paraoxonase has been determined to be due to the presence of isomers which differ in oxidation states.

The constant apoJ:paraoxonase mole ratio of 8.2 ± 2.1 in affinity-purified apoJ lipoproteins is indicative of a specific apoJ/paraoxonase interaction. The specificity of the interaction of apoJ with paraoxonase is further indicated by the ability of apoJ, when added to apoJ-depleted plasma, to combine with paraoxonase. While HPLC-purified apoJ reassociates with apoAI in a decreasing mole ratio, which approches that in the original plasma (Stuart et al., 1992), it reassociates with paraoxonase at a constant mole ratio, which is reasonably close to that found in the control plasma. The difference between apoJ's capacity to reassociate with plasma apoAI and with paraoxonase is significant: increasing the concentration of apoJ augments the binding of apoAI, while paraoxonase binding is linearly dependent on the apoJ concentration. On the basis of apoJ's known propensity to self-associate in solution, the data suggest that apoAI binds preferentially to apoJ oligomers while paraoxonase can associate equally with apoJ monomers and oligomers.

Further support for the specificity of the apoJ/paraoxonase interaction comes from direct binding studies. When purified apoJ is incubated with immobilized paraoxonase, specific and saturable binding occurs. In contrast, when purified apoE is incubated with immobilized paraoxonase as a control, no binding is detected. The apoJ binding curves revealed a variation in the apparent affinities of apoJ for paraoxonase as the paraoxonase coating concentration is increased. The affinity of apoJ for IgG has similarly been reported (Wilson & Easterbrook-Smith, 1992) to increase as the IgG concentration is increased. In the case of apoJ/paraoxonase, the phenomenon may result when higher concentrations of paraoxonase coating the plate decrease the paraoxonase–paraoxonase intermolecular distances, inducing conformational changes in the protein that allow it to bind with increasing affinity to apoJ. This is possible since subsaturating concentrations of paraoxonase were used to coat the microtiter plates in the direct binding studies, as is evidenced in the control binding study (Figure 9).

Furlong et al. (1991) previously reported that purified human paraoxonase exists as a doublet when analyzed by SDS–PAGE, although the molecular masses they calculated were 44.7 and 47.9 kDa. The small variation between their molecular mass values and that reported here, 44 kDa, may be due to differences in the conditions of SDS–PAGE analysis. Furlong et al. (1991) also found that both forms of paraoxonase have the same N-terminal sequence and enzymatic activity, and speculated that the appearance of two bands is due to carbohydrate heterogeneity (Furlong et al., 1991). The deglycosylation experiments performed in this study, however, ruled out this possibility. We conclude that the difference is due to the presence of multiple oxidation states of paraoxonase. ABD-F labeling of the two forms independently, in both the nonreduced and reduced states, indicates that the two forms of paraoxonase differ by one disulfide bond, present in paraoxonase-B between Cys_{42} and Cys_{284}. This evidence is supported by the protein's migration pattern on SDS–PAGE gels. The lower molecular mass species, containing the intramolecular disulfide bond, would be expected to form a compacted, faster migrating arrangement compared to the species containing all free sulfhydryls. Whether these different oxidation states have physiological significance remains to be determined.

Expression of human paraoxonase, unlike that of apoJ, is apparently limited to the liver. Northern blot analysis of RNA obtained from a variety of human tissues revealed the presence of paraoxonase-specific mRNA only in liver. In addition, Hassett (personal communication) found paraoxonase mRNA present in adult liver and not in fetal liver. These results, together with the fact that paraoxonase is not secreted from HepG2 cells in association with apoJ (Burkey et al., 1992), suggest that paraoxonase associates with apoJ in the plasma compartment. This hypothesis is supported by the reported paraoxonase/apoAI associations in plasma (Blatter et al., 1993; Gan et al., 1991), as well as the localization of paraoxonase in HDL species (Blatter et al., 1993; Don et al., 1975; Kitchen et al., 1973; Mackness et al., 1985).

The high degree of heterogeneity observed in isolated paraoxonase complexes has complicated the elucidation of the compositions of complexes containing paraoxonase. Molecular mass determinations of purified bovine paraoxonase preparations by gel filtration chromatography and sedimentation equilibrium indicate apparent molecular masses ranging from 70 kDa for a lipid-free preparation to 440 kDa for a lipid-rich preparation (Don et al., 1975; Kitchen et al., 1973). Two-dimensional nondenaturing gel analysis of plasma indicates that there is little overlap of apoJ/apoAI-HDL and apoJ/paraoxonase-HDL: apoJ-HDL which contain both apoJ and paraoxonase have molecular masses in the 90–140-kDa range, whereas the majority of apoJ-HDL which contain both apoJ and apoAI have molecular masses in the 350–400-kDa range (Stuart et al., 1992). This result further implicates a preference of apoAI for apoJ oligomers. Whether discrete apoJ/paraoxonase/apoAI particles exist remains to be determined.

Our knowledge of HDL metabolism is still quite limited, even with regard to the origin of HDL particles. This is due in part to the high degree of heterogeneity among HDL particles as well as a lack of specific function assigned to the various subclasses. ApoJ is primarily associated with lipid-poor subclasses of HDL that have $\alpha 2$ electrophoretic mobility (Stuart et al., 1992), whereas the bulk of paraoxonase occurs in the α-migrating HDL population, specifically in the HDL_2 subclass (Don et al., 1975; Kitchen et al., 1973; Mackness et al., 1985). The apoJ/paraoxonase species defines a discrete subclass of HDL which can now be used to "track" the metabolic path of these particles and contribute to our understanding of HDL metabolism in both normal and disease states.

Human serum paraoxonase is classified as an "A"-esterase, capable of hydrolyzing a number of carboxylic acid esters as well as the organophosphate paraoxon (*O,O*-diethyl-*O*-*p*-nitrophenyl phosphate) produced in mammals by microsomal oxidation of the insecticide parathion (Aldridge, 1953a,b). It demonstrates a genetic polymorphism for the hydrolysis of paraoxon. One allelic form of the enzyme has a high turnover number for paraoxon and the other allelic form, a low turnover number (Furlong et al., 1988). However, there is currently no known natural substrate for "A"-esterases in mammals. While there are indications in rabbits that an esterase may function in regulating blood cholesterol levels (Van Zutphen & Fox, 1977), the reaction catalyzed by the "A"-esterase bears resemblance to the hydrolytic activity characteristic of

phospholipase C and D (Mackness, 1989). Taking into account the catalytic activity of paraoxonase and the lipid transport role of apoJ, as well as the colocalization of apoJ and paraoxonase in HDL, it is possible that the two proteins, along with apoAI, play a concerted role in reverse cholesterol transport.

Equally exciting is the possibility that the apoJ–paraoxonase complex will be a useful indicator of the susceptibility to atherosclerosis. In human serum, where much of the paraoxonase is HDL-associated, the enzyme activity is significantly reduced in postmyocardial infarct patients (McElveen et al., 1986). Paraoxonase activity in patients with "Fish eye" disease, characterized by a 90% decrease in HDL-cholesterol and apoAI and apoAII, is reduced by approximately 90% compared to controls (Mackness et al., 1987). A patient with Tangiers disease, characterized by greatly reduced levels of apoAI and apoAII, lacked detectable levels of paraoxonase activity (Mackness, 1989). In a study conducted in Hungary (Szabo et al., 1987), the progeny of patients who had experienced early myocardial infarction had much lower levels of paraoxonase activity compared to "healthy" progeny. Taken together, these findings lead to the prediction that low paraoxonase activity is associated with premature myocardial infarction. Since apoJ is positively correlated with plasma triglyceride and cholesterol (Jenkins et al., 1990), and has recently been detected in atherosclerotic lesions (Aronow et al., 1993), the possibility exists that an increased apoJ: paraoxonase ratio (due to increased apoJ levels and decreased paraoxonase levels) may be an indicator of vascular damage. Careful analysis of all of these connections should resolve the question of whether the apoJ–paraoxonase complex may indeed be useful as a predictive index of the predisposition to develop atherosclerosis and heart disease.

ACKNOWLEDGMENT

We appreciate the helpful suggestions provided by Drs. Terence Kirley and Earl Wallick, and the expert protein sequencing and amino acid analysis provided by Ms. Cleris Gil.

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