Purification of Rabbit and Human Serum Paraoxonase†

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ABSTRACT: Rabbit serum paraoxonase/arylesterase has been purified to homogeneity by Cibacron Blue–agarose chromatography, gel filtration, DEAE-Trisacryl M chromatography, and preparative SDS gel electrophoresis. Renaturation (Copeland et al., 1982) and activity staining of the enzyme resolved by SDS gel electrophoresis allowed for identification and purification of paraoxonase. Two bands of active enzyme were purified by this procedure (35 000 and 38 000). Enzyme electroeluted from the preparative gels was reanalyzed by analytical SDS gel electrophoresis, and two higher molecular weight bands (43 000 and 48 000) were observed in addition to the original bands. This suggested that repeat electrophoresis resulted in an unfolding or other modification and slower migration of some of the purified protein. The lower mobility bands stained weakly for paraoxonase activity in preparative gels. Bands of each molecular weight species were electroblotted onto PVDF membranes and sequenced. The gas-phase sequence analysis showed that both the active bands and apparent molecular weight bands had identical amino-terminal sequences. Amino acid analysis of the four electrophoretic components from PVDF membranes also indicated compositional similarity. The amino-terminal sequences are typical of the leader sequences of secreted proteins. Human serum paraoxonase was purified by a similar procedure, and ten residues of the amino terminus were sequenced by gas-phase procedures. One amino acid difference between the first ten residues of human and rabbit was observed.

Parathion is an organophosphorus insecticide that undergoes activation through oxidative desulfuration by the cytochrome P-450 system (Murphy, 1980). The active metabolite paraoxon can be inactivated (hydrolyzed) by the serum enzyme paraoxonase. Serum paraoxonase levels vary widely in different animal species [reviewed in Geldmacher-von Mallinckrodt and Diepgen (1988)]. Various correlative data suggest that high serum paraoxonase levels provide protection against parathion poisoning. Paraoxonase levels in birds (Machin et al., 1976; Brealey et al., 1980), humans (Ghezzo et al., 1977), and rats and rabbits (Costa et al., 1987) correlate with sensitivity or resistance to inhibition of cholinesterase by parathion or paraoxon. Recently, we found that paraoxonase also hydrolyzes chlorpyrifos oxon, the active toxic metabolite of chlorpyrifos, and that sera from rabbits exhibit very much higher rates of chlorpyrifos oxon hydrolysis than sera from rats (Costa et al., 1987). These results agree with the correspondingly higher LD_{50} value for reported chlorpyrifos toxicity in rabbits compared with rats (McCollister et al., 1974).

Experiments have directly addressed the ability of paraoxonase to protect from organophosphorus toxicity. Main (1956) injected partially purified rabbit paraoxonase iv into rats and followed with an iv paraoxon challenge. Paraoxonase was demonstrated to provide protection from paraoxon toxicity. Our recent experiments confirm and extend these observations. Injection of rabbit paraoxonase into rats provided protection from paraoxon toxicity and even greater protection against chlorpyrifos oxon toxicity (Costa et al., 1990).

In humans, paraoxonase demonstrates a genetically determined, substrate-dependent polymorphism [reviewed by Geldmacher-von Mallinckrodt and Diepgen (1988)]. One allelic form of the enzyme hydrolyzes paraoxon with a low turnover number and the other with a high turnover number. Both forms of the enzyme appear to hydrolyze chlorpyrifos oxon (Furlong et al., 1988, 1989) and phenylacetate (Eckerson et al., 1983; LaDu & Eckerson, 1984; Gan et al., 1991; Smolen et al., 1991) with the same or nearly the same turnover number. In addition to the polymorphism, variable expression of the allelic forms of the enzyme appears to govern overall serum enzyme levels (Furlong et al., 1988, 1989).

Many attempts have been made to purify serum paraoxonase; however, purification to homogeneity has proven difficult, probably because paroxonase is intimately associated with the high-density lipoprotein complex (Kitchen et al., 1973; Don et al., 1975; Mackness et al., 1985; Zimmerman & Brown, 1986). Recently, Gan et al. (1991) reported the purification of human paraoxonase to homogeneity. In this report, we describe purification of rabbit and human paraoxonase to homogeneity and the identical amino-terminal sequence of electrophoretically different forms of the enzyme in rabbit. Amino-terminal sequence data for human paraoxonase are also reported.

Materials and Methods

Materials. Chlorpyrifos oxon was kindly provided by Dow Chemical Co. (Midland, MI). Spectral analysis indicated little contamination by 3,5,6-trichloro-2-pyridinol. Paraoxon was obtained from ICN K & K Labs (Plainview, NY). Material from other suppliers was found to contain unacceptable levels of *p*-nitrophenol. *Caution, these organophosphates are highly toxic nerve poisons.* They have potent anticholinesterase action and can be absorbed percutaneously. Therefore, they should be handled with great care. Waste from the assays can be inactivated by strong NaOH solutions. Cibacron Blue 3GA–agarose type 3000 was from Sigma Chemical Co. (St. Louis, MO). DEAE-Trisacryl M was from IBF Biotechnics (Savage, MD). Immobilon P membranes were obtained from Millipore Corp., San Francisco, CA.

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Cibacron Blue 3GA–Agarose Chromatography. Rabbit serum was fractionated on Cibacron Blue 3GA–agarose as described by Eckerson et al. (1983), except that the heparin–$MgSO_4$ precipitation step was eliminated.

Protein Concentration. When necessary, fractions containing paraoxonase activity were pooled and concentrated in Amicon stirred cells fitted with YM-10 membranes.

Gel Filtration Chromatography. To remove excess detergent and/or salts, concentrated pools of fractions were passed through Sephadex G-75 columns equilibrated with 15 mM Tris-HCl, pH 8.0 (and 1 mM $CaCl_2$ if deoxycholate was not present in the sample). The fractions containing paraoxonase activity were pooled (brought to 1 mM $CaCl_2$ if necessary) and reconcentrated if necessary in an Amicon stirred cell.

DEAE-Trisacryl M Chromatography. DEAE-Trisacryl M columns were equilibrated with 15 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, and 0.1% Nonidet P-40. The desalted, concentrated paraoxonase pool from the gel filtration columns was adjusted to equilibration buffer conditions by the addition of 25% NP-40 to obtain the concentrations noted above. The concentrated sample was loaded onto the column, which was then rinsed to baseline OD with equilibration buffer (3 resin bed volumes). The adsorbed protein was eluted with a linear gradient from 0 to 0.125 M NaCl (50 resin bed volumes) in column equilibration buffer.

Preparative SDS Gel Electrophoresis. Gradient pore (4%–18%) gels (16 cm × 16 cm × 0.75 mm thick) were loaded with partially purified paraoxonase across the entire width of the gel and resolved in a gel apparatus (Riverside Scientific Enterprises, Winslow, WA, Model 7.5). Following electrophoresis, the gel was renatured by the procedure of Copeland et al. (1982), and bands containing paraoxonase activity were located by staining either 1-cm strips cut from each side of the gel or the entire gel with the β-naphthylacetate activity stain procedure described below. Bands containing enzymatic activity were cut from the gel with a clean razor blade and electroeluted as described below.

Analytical SDS Gel Electrophoresis. Analytical gels were 10 cm high × 8 cm wide gradient pore gels (4%–18%) with the Laemmli (1970) resolving gel buffer system. To locate protein bands with paraoxonase activity, the gels were stained with the β-naphthylacetate activity stain described below before staining with Coomassie blue. Gels were stained for protein with either Coomassie blue or the silver-staining procedure of Oakley et al. (1980).

Electroblotting of Proteins. Proteins resolved on "preparative minigels" (10 cm × 8 cm × 0.75 cm) were electroblotted onto polyvinylidene difluoride (PVDF) membranes as described by Matsudaira (1987). After electroblotting, a 1-cm strip was cut from one side of the membrane and stained for activity after renaturing. The remainder of the blot was stained with Coomassie blue R-250 (Matsudaira, 1987). Realignment of the activity-stained strip with the Coomassie blue stained membrane identified the enzymatically active protein bands.

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).

Enzyme Activity Stains. Following either ionic and nonionic detergent preparative or analytical gel electrophoresis, and isoelectric focusing, proteins were renatured before staining by briefly rinsing the gel (1–2 min) in staining buffer (Copeland et al., 1982). Bands containing paraoxonase activity were stained either by a modification of the α-naphthylacetate staining procedure described by Paul and Fottrell (1961) or by a direct paraoxon activity stain developed in this study. The buffer used for both stains was 100 mM Tris-HCl, pH 8.5, which contained 10 mM $CaCl_2$ and 3.5 M NaCl. For the β-naphthylacetate staining procedure, Fast blue RR salt (Holmes & Masters, 1967) was used in place of Fast blue B. β-Naphthylacetate was used in place of α-naphthylacetate (Holmes & Masters, 1967). Esterase activity appeared as red bands.

To stain directly for paraoxon hydrolysis, the staining buffer contained 1.2 mM paraoxon (diethyl *p*-nitrophenyl phosphate). The position of paraoxonase was readily visible as yellow bands of *p*-nitrophenol which diffused with time (observe caution noted under Materials and Methods).

Preparative Isoelectric Focusing. Desalted, concentrated paraoxonase was absorbed to a standard sample wick and resolved on a preparative agarose isoelectric focusing gel (pH 3.5–10.0, 110 mm × 125 mm × 2 mm). The IEF gel which contained 1% Isogel–agarose, 2.5% ampholytes (pH 3.5–10), 10% *d*-sorbitol, and 0.25% NP-40 was cast onto GelBond film. The sample wick was applied the length of the gel 2 cm from the cathode. The sample was allowed to migrate into the gel at 2 W for 20 min; then the sample application wick was removed, and the gel was allowed to run up to limits of 500 V at 10 W for 90 min. Paraoxonase was visualized as a red band at the acidic end of the gel by using the β-naphthylacetate activity stain. The activity was also located as a yellow band with the paraoxon activity stain on a 1-cm strip cut crom the same gel.

Nonionic Detergent Gel Electrophoresis. The buffer system of Ornstein (1964) and Davis (1964) was augmented with 0.25% Nonidet P-40. Gradient pore acrylamide gels of 4%–16% eliminated stacking gel requirements for both analytical and preparative gels.

Electroelution. Protein bands resolved by SDS gel electrophoresis were cut from gel, passed through a syringe to a macerate the gel, electroeluted in a device preparated from a disposable 11-mL Econo column (Bio-Rad). A male banana plug connector with a platinum electrode was fitted to the column cap which contained a gas vent hole. The column outlet was fitted with a 0.25-in. polypropylene adapter and covered with boiled (Maniatis et al., 1982) 10-mm Spectrapore dialysis tubing (12 000–14 000 cutoff) closed with a 14-mm Spectrapore closure. A small ring of Tygon tubing served to clamp the top of the dialysis tubing against the adapter outlet. A cooled chamber fitted with a platinum electrode served as the lower buffer reservoir. Protein from 5 mL of macerated gel could be electroeluted into 200 μL of SDS running buffer. Electroelution was usually for 2 h at 100 V (~5 mA).

Amino Acid Analysis and Edman Degradation. Phenylthiocarbamyl amino acid analysis was performed as previously described (West & Crabb, 1990), using an Applied Biosystems automatic system (Models 420/430/920). Manual HCl vapor-phase hydrolysis was performed at 150 °C for 1 h (Tarr, 1986) in the presence of phenol. Following vapor-phase hydrolysis of electroblotted and Coomassie blue stained samples on PVDF membranes, amino acids were extracted 2–3 times with 50 mL of 30% acetonitrile and applied to the amino acid analyzer. Microsequence analysis (Crabb et al., 1988) of PVDF-immobilized samples was performed with an Applied Biosystems gas-phase sequencer (Model 470) and an on-line phenylthiohydantoin analyzer (Model 120) by using the modified 01CPVD sequencer program (Speicher, 1989). Initial sequencer yields of 6–37 pmol and repetitive yields greater than 91% were obtained.

Table I: Purification of Rabbit Paraoxonase

step	vol (mL)	act.[a] units/mL	act.[a] total units	[protein] (mg/mL)	sp act. (units/mg)	purification (x-fold)[b]	recovery (%)
whole serum	150	3.61	542.1	62.5	0.057		100
agarose blue	33	10.56	348.6	23.6	0.45	7.9	64
Sephadex G-75	32	7.78	249.0	12.0	0.65	11.4	45
DEAE-Trisacryl M	20	3.05	61.1	1.7	1.8	31.6	11
Sephadex G-75	10	16.68	166.8	4.0	4.17	73.1	31

[a] Units = μmol/min. [b] A 73-fold purification of paraoxonase was obtained with a 31% yield. A gel electrophoretic analysis of the purification is shown in Figure 1.

Protein Determinations. Protein concentrations were determined by the bicinchoninic acid procedure of Smith et al. (1985).

Results

Purification of Rabbit Serum Paraoxonase

Our objective was to obtain rabbit paraoxonase of sufficient purity for biochemical charaterization, protein sequence analysis, and oligonucleotide probe design for cloning. Four column steps routinely provided 60–100-fold purification.

Reactive Blue Agarose Chromatography. Rabbit serum (150 mL) was diluted with 150 mL of 25 mM Tris-HCl, pH 8.0, containing 2 M NaCl, 0.5 mM $CaCl_2$, and 2.5 μM EDTA. The diluted serum was fractionated on a 200-mL resin bed volume (4.7 cm × 11 cm) Cibacron Blue 3GA–agarose column (Eckerson et al., 1983) as described under Materials and Methods. This step resulted in a 7.9-fold purification (Table I). The fractions containing paraoxonase activity were concentrated by ultrafiltration and desalted on a Sephadex G-75 column as described under Materials and Methods (Figure 1A). The gel filtration/desalting step resulted in an additional 1.4-fold purification of paraoxonase (Table I).

DEAE-Trisacryl M Chromatography. The pooled, concentrated, and desalted paraoxonase-containing fractions from the Cibacron Blue 3GA–agarose chromatography step were further fractionated by detergent DEAE-Trisacryl M chromatography (Figure 1B). Fractions containing paraoxonase activity were pooled, concentrated, and desalted on a Sephadex G-75 column (Figure 1C). (Sephadex G-200 should be used at this point if significant albumin is present at this step of the purification.) The gel filtration step removes excess detergent which inhibits enzyme activity. The purification is summarized in Table I.

Activity-Stain Analysis of Purified Paraoxoase. The ability to reactivate and activity-stain paraoxonase following SDS gel electrophoresis has been crucial to the purification efforts. Incubation of gels with substrate localized activity and allowed assessment of the relationship of particular protein bands to activity. Activity- and protein-stained analytical SDS gels of the DEAE-Trisacryl M purified protein from two different preparations showed two active bands at 35 000 and 38 000 (Figure 2). The gel was first stained with β-naphthylacetate and then with Coomassie blue. Gels could also be stained with paraoxon; however, the color developed more slowly and the *p*-nitrophenol diffused with time (data not shown). Both activity stains identified the same protein bands at 35 000 and 38 000. A 73-fold purification of paraoxonase was achieved through the second Sephadex G-75 column, with an overall recovery of 31% (Table I).

Preparative SDS Gel Electrophoresis. DEAE-Trisacryl M purified paraoxonase (4 mg, in 0.5 mL) was resolved by preparative SDS gel electrophoresis as described under Materials and Methods. Following resolution of the protein bands, 1.5-cm strips of gel were cut from each side of the gel and activity-stained with β-naphthylacetate. Two bands of activity were observed with the β-naphthylacetate stain. The stained strips were used to align the gel for excision of the active paraoxonase bands, which were then electroeluted. Both of

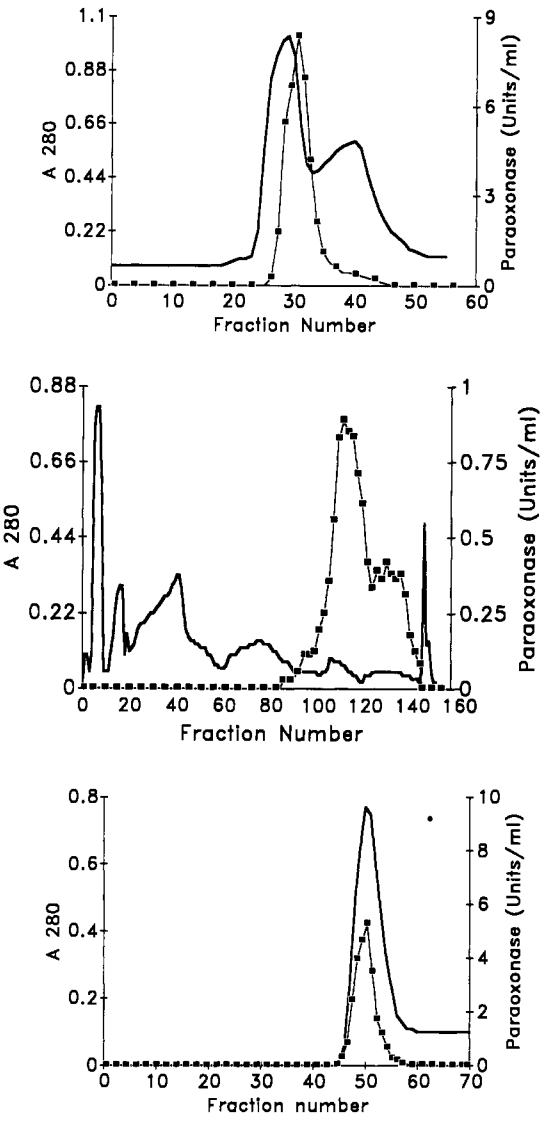

FIGURE 1: (A, top) Sephadex G-75 gel filtration of concentrated paraoxonase-containing fractions from the Cibacron Blue column. Solid line, relative A_{280}; boxed line, paraoxonase activity. (B, middle) DEAE-Trisacryl M fractionation of the pooled paraoxonase fraction from the Sephadex G-75 column. The concentrated paraoxonase sample from the gel filtration column (30 mL) was fractionated on a 20-mL resin bed volume DEAE-Trisacryl M column (1.8 cm × 8 cm) as described under Material and Methods. Solid line, relative A_{280}; boxed line, paraoxonase activity. (C, bottom) Second Sephadex G-75 gel filtration. The concentrated sample from the DEAE-Trisacryl M pool (32 mL) was desalted/fractionated on a 1.3-L (4 cm × 103 cm) column of Sephadex G-75 as described under Materials and Methods. The fractions containing paraoxonase activity were concentrated. Solid line, relative A_{280}; boxed line, paraoxonase activity.

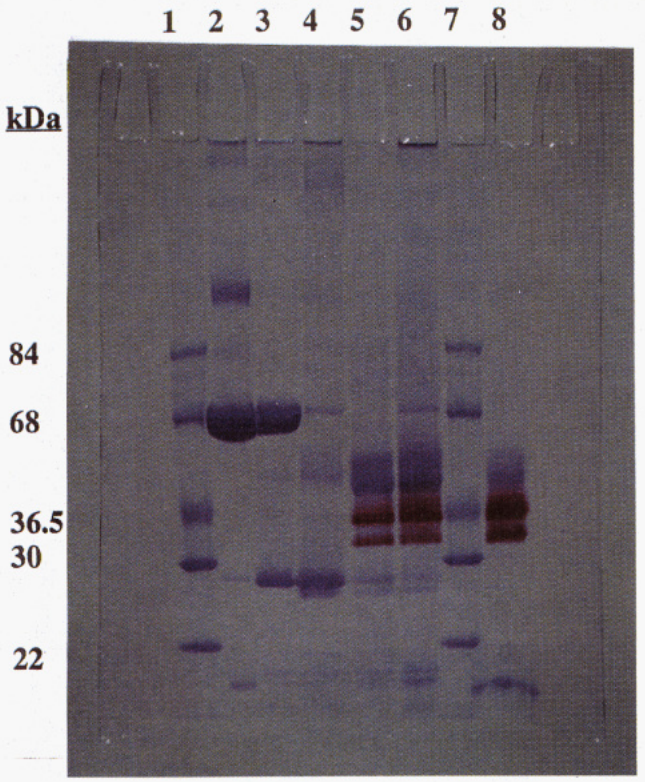

FIGURE 2: Analytical SDS gel electrophoretic analysis of fractions from paraoxonase purification steps: lanes 1 and 7, molecular weight markers at 84 000 (fructose-6-phosphate kinase), 68 000 (bovine serum albumin), 36 500 (lactate dehydrogenase), 30 000 (carbonic anhydrase), and 22 000 (trypsin inhibitor); lane 2, whole rabbit serum (15.6 μg); lane 3, Cibacron Blue 3GA–agarose pool (40 μg); lane 4, Sephadex G-75 pool (24 μg); lane 5, DEAE-Trisacryl M pool (13 μg); lane 6, second Sephadex G-75 pool (16 μg); lane 8, DEAE-Trisacryl M fraction from another purification (27 μg). The gel was first stained with the β-naphthylacetate activity stain, followed by Coomassie blue. The activity stained bands are shown in red.

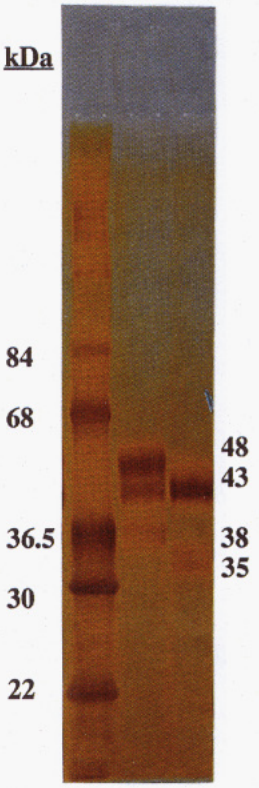

FIGURE 3: Silver-stained analytical SDS gel analysis of paraoxonase electroeluted from the preparative SDS gel: left lane, molecular weight standards; center lane, high molecular weight electroeluted paraoxonase band (38 000); right lane, low molecular weight electroeluted paraoxonase band (35 000).

the active bands were also stained with the diffusible paraoxon stain in analytical gels of the same material (not shown).

Changes of Electrophoretic Mobility of Purified Paraoxonase. Analysis of the gel-purified, electroeluted paraoxonase by SDS analytical gels produced a curious result (Figure 3). Each active band (~35 000 and 38 000) produced a band in the original position and also another of apparent higher molecular weight. The bands in the original position identified by activity stain remained active, while the new bands at higher molecular weights did not stain for activity, only for protein with Coomassie blue. The inactive bands migrated at positions of ~43 000 and 48 000.

Alternative Electrophoretic Purification Procedure. Since we previously had success in renaturing proteins following denaturing two-dimensional gel electrophoresis (Copeland et al., 1982), we felt that if enzymatic activity could be restored following isoelectric focusing in the presence of detergent, it should be possible to excise the band of activity and further resolve the enzyme by SDS gel electrophoresis. If the enzymatic activity could again be renatured, it would be possible to excise the active protein band and recover the enzyme by electroelution. Such an approach would be equivalent to a preparative two-dimensional gel electrophoresis procedure.

Paraoxonase activity was resolved by preparative isoelectric focusing in the presence of NP-40, as described under Materials and Methods. A strip of the gel was removed for direct staining with paraoxon, and the remainder of the gel was stained with the β-naphthylacetate procedure. Figure 4A shows that it was indeed possible to renature esterase activity following isoelectric focusing in the presence of the neutral detergent NP-40. The strip of gel activity stained with paraoxon as a substrate showed a diffusible yellow band that coincided with the same band responsible for β-naphthylacetate hydrolysis.

The band staining red for paraoxonase activity was excised with a clean razor blade, macerated by passage through a 10-mL syringe fitted with an 18-gauge needle, and loaded directly onto a 16 cm × 16 cm × 0.75 mm gradient pore (4%–18%) preparative SDS gel. Following electrophoresis, the entire gel was stained with the β-naphthylacetate activity stain. Figure 4b shows that it was also possible to renature the β-naphthylacetate-hydrolyzing activity following SDS gel electrophoresis. Two bands of paraoxonase activity were observed. Each of the active bands was carefully excised from the gel and rerun on separate gradient pore SDS gels. Following resolution in the second preparative gels, bands containing activity were again located with the β-naphthylacetate stain, excised with a clean razor blade, macerated by passage through a syringe, and electroeluted in the device described under Materials and Methods. The active bands also ran as single active bands on the second preparative SDS gels. Figure 4C shows the activity stain of the gel with the higher molecular weight band. Following resolution on the second preparative SDS gels, the active bands were excised, electroeluted, and analyzed by SDS gel electrophoresis (Figure 4D). The analytical gel showed that the most of the protein from the active bands dropped back to the positions of the inactive protein bands with higher molecular weights as had been seen previously.

Amino-Terminal Sequencing of Rabbit Paraoxonase. The curious observation of the shift in protein band position following purification was investigated by separating the para-

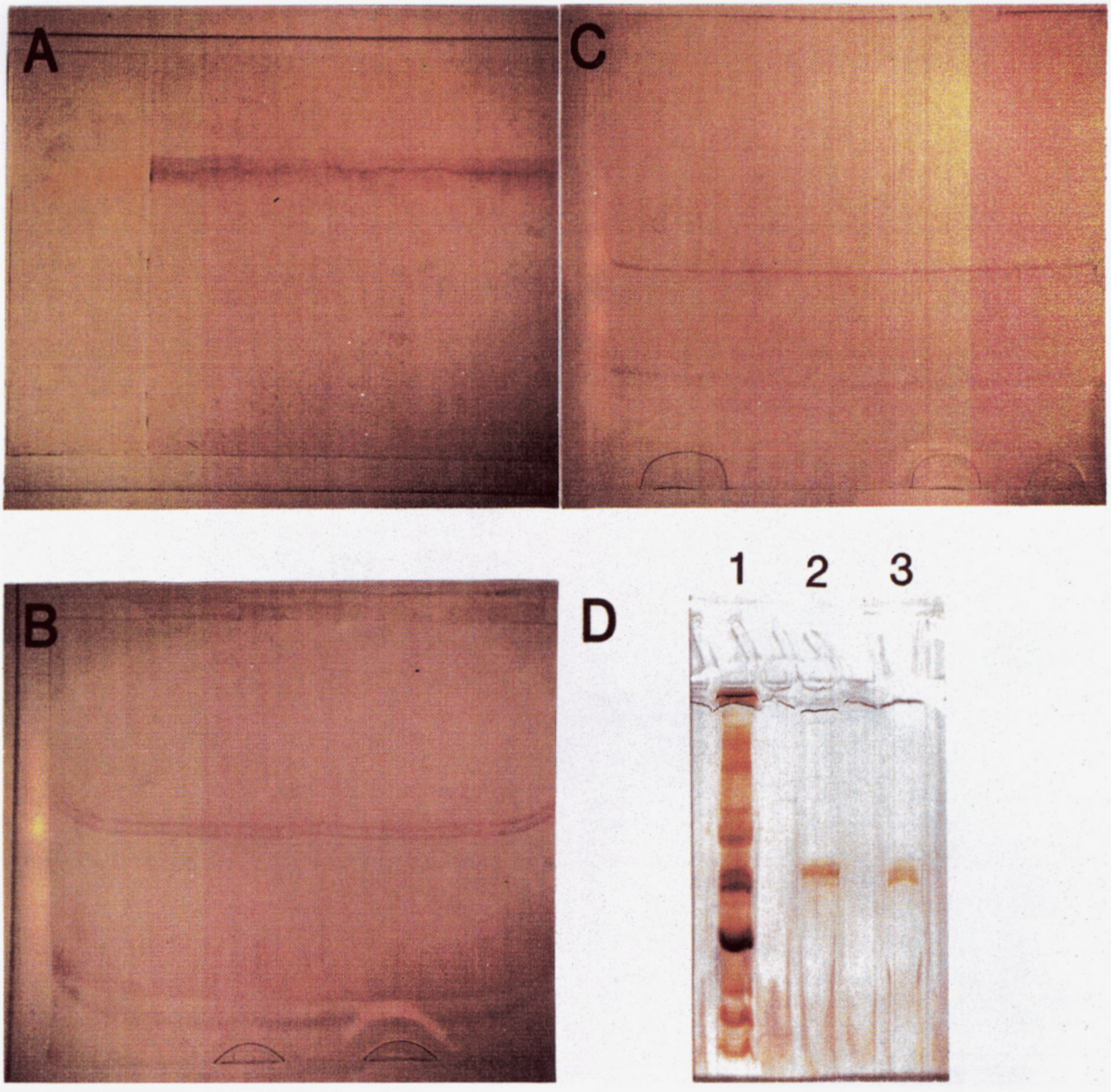

FIGURE 4: (A) Preparative isoelectric focusing gel after a 1-cm strip was stained with paraoxon and the remainder of the gel was stained with β-naphthylacetate. (B) β-Naphthylacetate-stained SDS preparative gel purification of paraoxonase from the preparative IEF step. (C) β-Naphthylacetate-stained SDS preparative gel of the high molecular weight band excised from the gel in panel A and rerun on a second SDS preparative gel. (D) Analytical SDS gel analysis of paraoxonase activity-stained bands electroeluted from the second preparative gels: lane 1, starting material (desalted fraction from the Cibacron Blue 3GA–agarose step); lane 2, high molecular weight paraoxonase band; lane 3, low molecular weight paraoxonase band. Gels were run as described under Materials and Methods.

oxonase purified through the DEAE-Trisacryl M chromatography step on a minipreparative SDS gel. The sample (216 μg in 120 μL) was applied to the full width of the 8-cm gel. Following electrophoresis, a 1-cm gel slice was stained for activity with β-naphthylacetate and then for protein with Coomassie blue (Figure 5). The remainder of the gel was electroblotted onto a Immobilon P (PVDF) membrane. A 1-cm strip of the membrane was activity-stained and the remainder stained with Coomassie blue. The activity-stained strip identified the two bands with esterase activity. All four bands were cut from the Coomassie blue stained membrane with a clean razor blade and sequenced on the Applied Biosystems gas-phase protein sequencer. Each of the four bands had the same amino-terminal sequence (AKLTALTLLGLGLALFDGQK). This suggested that the bands of apparent higher molecular weight that arose from the homogeneous gel-purified bands of lower molecular weight were probably the result of protein denaturing and unfolding or loss of material associated with the protein that would affect its mobility. (Twenty cycles of sequence were determined for the 35 000 and 38 000 bands, 16 for the 43 000 band, and 13 for the 48 000 band.) Determination of the amino acid compositions of each band also indicated compositional similarity between the four bands (Table II). The amino acid compositions are in reasonable agreement with the amino acid composition deduced from the rabbit paraoxonase cDNA sequence (Hassett et al., 1991). With high protein loads on preparative gels, the higher molecular weight bands stained faintly with the β-naphthylacetate staining procedure.

Purification and Amino-Terminal Sequence of Human Paraoxonase

A small quantity of human paraoxonase was purified for amino-terminal sequencing. Essentially the same chromato-

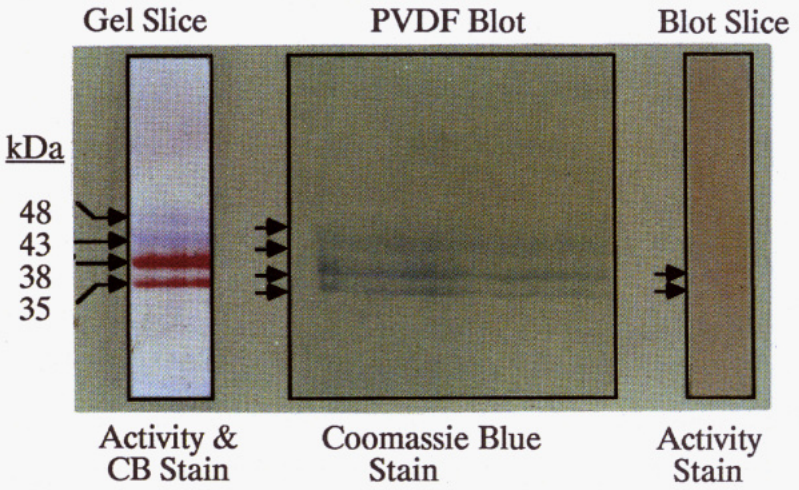

FIGURE 5: Minipreparative gel strip and electroblot: (left) 1-cm strip of the minipreparative gel stained first with β-naphthylacetate and then with Coomassie blue; (center) Immobilon electroblot stained with the Matsudaira Coomassie blue procedure; (right) 1-cm strip of the Immobilon electroblot stained with the β-naphthylacetate stain.

Table II: Paraoxonase Amino Acid Analysis on PVDF[a]

amino acid	deduced from sequence	electroblotted bands[b] 56 000	49 000	37 000	36 000
D	12.54	11.10	12.84	13.61	11.96
E	7.12	10.09	9.81	8.55	9.00
S	6.84	9.27	8.57	7.43	8.28
G	6.55	12.04	10.52	9.36	10.82
H	3.70	1.40	1.50	1.89	1.69
R	1.71	1.83	1.33	1.41	1.72
T	5.70	4.48	4.57	3.94	4.30
A	5.70	7.52	7.10	6.96	6.92
P	5.98	5.39	5.70	5.71	5.50
Y	4.27	4.06	4.09	4.32	4.36
V	8.83	7.52	7.59	6.92	7.65
M	1.71	0.20	0.25	0.47	0.34
I	5.41	4.47	4.49	4.65	4.67
L	11.97	12.42	12.67	13.53	13.15
F	5.13	4.58	4.79	5.13	4.97
K	6.84	3.65	3.98	5.04	4.58
n		4	4	5	5
av μg of protein analyzed		0.19	0.64	0.62	0.29

[a] Values are expressed in mole percent. [b] From Figure 5.

graphic procedures were used for the human enzyme as were used for purifying rabbit paraoxonase. Table III summarizes a typical purification using agarose blue chromatography followed by Sephadex G-200 gel filtration, detergent DEAE-Trisacryl M chromatography, and desalting/gel filtration on a Sephadex G-75 column. Since we have only occasionally been successful in renaturing human paraoxonase following SDS gel electrophoresis, concentrated enzyme from the final Sephadex G-75 column was resolved in a 4%–16% NP-40-containing Ornstein–Davis minipreparative gel. Activity was located by staining 1-cm strips cut from each edge of the gel with β-naphthylacetate activity-staining procedure. Active enzyme was electroeluted and further purified by SDS gel electrophoresis in four lanes of an 8 × 10 cm SDS 4%–16% gradient pore minigel. The protein bands were electroblotted to a PVDF membrane and stained. Two bands were visible (44 700 and 47 900). These bands were excised and sequenced by the gas-phase procedures. The first ten amino-terminal residues were identified (AKLIALTLLG). One difference between the rabbit and human amino-terminal sequences was observed at position 4. The observed difference has been confirmed by cDNA sequencing (Hassett et al., 1991).

Hydrolysis of Other Substrates by Paraoxonase. We routinely have used phenylacetate as a substrate for initial monitoring of column fractionation procedures. The fractionation pattern was then verified with paraoxon and occasionally with chlorpyrifos oxon as substrates. In the hundreds of column fractionation procedures that we have carried out over the past 8 years [see also Ortigoza-Ferado et al. (1984) and Furlong et al. (1988, 1989)], all three activities have copurified (data not shown) with both rabbit and human paraoxonase. As noted above, in all electrophoretic procedures we have used, paraoxon- and β-naphthylacetate-hydrolyzing activities have comigrated.

Discussion

There has been some controversy regarding whether paraoxonase catalyzes the hydrolysis of substrates such as phenylacetate and β-naphthylacetate. Mackness et al. (1987, 1989) claimed that human serum contained two paraoxonases and three arylesterases without cross-substrate specificity. Haas and Geldmacher-von Mallinckrodt (1990), on the basis of electrophoretic studies with crude sera, reported that paraoxonase could be resolved from arylesterase. On the other hand, the work of LaDu and co-workers (Eckerson et al., 1983; LaDu & Eckerson, 1984) and our own previous work (Furlong et al., 1988, 1989) indicated that both phenylacetate and

Table III: Purification of Human Paraoxonase

step	vol (mL)	act.[a] units/mL	act.[a] total units	[protein] (mg/mL)	sp act. (units/mg)	purification (*x*-fold)[b]	recovery (%)
whole serum	12.5	1.44	18.0	62.0	0.023		100
agarose blue	12.5	1.31	16.4	3.1	0.42	18.1	91
Sephadex G-200	65	0.092	6.0	0.54	0.17	7.4	33
DEAE-Trisacryl M	5.9	0.46	2.76	0.49	0.93	40.4	15
Sephadex G-75 concentrate	1.9	1.72	3.27	1.2	1.43	62.1	18

[a] Units = μmol/min. [b] A 62-fold purification of paraoxonase was obtained with an 18% yield.

paraoxon were hydrolyzed by the same enzyme which also hydrolyzed chlorpyrifos oxon. To resolve this question and at the same time generate protein for sequencing and oligonucleotide probe design, we took the approach of purifying rabbit paraoxonase, which is much more active than human paraoxonase (Costa et al., 1990). The development of activity stains for paraoxon and β-naphthylacetate hydrolysis allowed us to verify that the same protein bands hydrolyze both substrates. While this paper was in preparation, Gan et al. (1991) and Smolen et al. (1991) reported the purification to homogeneity and characterization of human paraoxonase. In agreement with the experiments reported here, their purification and kinetic studies indicated that paraoxon hydrolyzes phenylacetate, paraoxon, and β-naphthylacetate.

The reason for the appearance of two bands of activity is not known. It may be due to differences in carbohydrate content. Gan et al. (1991) report that human paraoxonase contains 15.8% carbohydrate. Sequence analysis (Hassett et al., 1991) indicates five potential N-glycosylation sites in rabbit paraoxonase and four in human. The molecular weight determinations by SDS gel electrophoresis are complicated by the shifting of bands following purification. The active rabbit bands at 35 000 and 38 000 shift to 43 000 and 48 000 when electroeluted and reanalyzed. The human paraoxonase bands are at 44 700 and 47 900, or at virtually the identical positions of the "denatured" rabbit enzyme. Gan et al. (1991) report a minimum molecular weight of 43 000 for human paraoxonase (37 000 without carbohydrate). Their molecular weight estimates as well as those reported here are in reasonable agreement with a molecular weight of 39 617 calculated from the deduced human paraoxonase sequence without allowance for contribution from glycosylation (Hassett et al., 1991). Zimmerman and Brown (1986) reported the presence of two closely spaced bands of 40 000–45 000 and 47 000–54 000 in partially purified rabbit preparations, in agreement with the size of the denatured rabbit paraoxonase that we have observed.

The starting specific activity of the human serum paraoxonase in this study was higher than that reported in the purification described by Gan et al. (1990). They achieved a higher fold purification with the agarose blue step than reported here. Their column steps provide a higher degree of purification than the column steps reported here and should be useful for further purification studies. The main aim of the studies reported here was to provide solid evidence that the protein bands to be sequenced were indeed paraoxonase for purposes of probe design and cloning. The use of renaturation of activity in gels achieved this aim. One important difference between the two different purification procedures is that the preparation described here provides protein which can be sequenced from the amino terminus, whereas the preparation described by Gan et al. (1990) was reported to have a blocked amino terminus. The amino acid composition of their human paraoxonase agrees reasonably well with the composition derived from the human cDNA sequence (Hassett et al., 1991).

Human paraoxonase appears to be more easily denatured by SDS than the rabbit enzyme. We have had only occasional success in activity-staining human paraoxonase following SDS gel electrophoresis, even in the presence of dilute SDS. The reason for the denatured forms of rabbit and human paraoxonase running with higher apparent molecular weights is unknown.

The difficulties encountered in many previous attempts by our laboratory and others to purify serum paraoxonases from different sources [reviewed in Geldmacher-von Mallinckrodt and Diepgen (1988)] stem in part from its apparent association with the HDL complex (Mackness et al., 1985), requiring detergents for purification (Gan et al., 1991). Further complicating the purification is the probable further denaturation or other modification of homogeneous protein. Protein which has been purified through four column chromatographic steps followed by preparative isoelectric focusing and two sequential preparative SDS gel electrophoresis steps exhibits a protein band at the molecular weight where the preparation stains for active esterase but also a more intense band that migrates slower than the active band. In preparative SDS gels, it is possible for a protein of lower molecular weight to trail and contaminate bands of higher molecular weight but not for a band of higher molecular weight to contaminate bands of lower molecular weights. This observation initially was perplexing. Obtaining the same amino-terminal sequence from the two active paraoxonase bands and the corresponding slower running bands generated from the pure active bands suggested that further unfolding of protein following purification is the simplest explanation for this observation.

The hydrophobic amino-terminal sequence is typical of the leader sequence of secreted proteins (von Heijne, 1985). Since this hydrophobic sequence is apparently not removed following secretion, it may serve to anchor the protein to the HDL complex.

The physiological substrate of paraoxonase remains unknown. The results reported here support the contention of Gan et al. (1991) that a separate classification for paraoxonase (Webb, 1989) is not necessary. When the endogenous substrate(s) is (are) identified, a new name should follow; until then, the designation paraoxonase/arylesterase (EC 3.1.1.2) should suffice.

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