CALCIUM BINDING BY HUMAN AND RABBIT SERUM PARAOXONASES
Structural Stability and Enzymatic Activity

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Equilibrium dialysis and Scatchard plots were used to establish that human and rabbit paraoxonases both have two calcium binding sites. Independent-site and stepwise constant analyses were used to calculate a higher affinity site (Kd1) of 3.8 ± 0.9 x 10^-7 M for human A paraoxonase, and 1.4 ± 0.5 x 10^-8 M for rabbit paraoxonase, and a lower affinity site (Kd2) of 6.6 ± 1.2 x 10^-6 M for human A paraoxonase, and 5.3 ± 0.94 x 10^-6 M for rabbit paraoxonase. In both species, the higher affinity sites were found to be essential to maintain hydrolytic activity; complete removal of calcium led to irreversible inactivation. The lower affinity sites were required for catalytic activity, and their binding of calcium was reversible. Experimentally estimated values of Kd2 based on the concentration of calcium required to obtain half the maximum enzymatic activity were 3 μM for human A and B paraoxonases, and also in the order of 3 μM for rabbit paraoxonase, using three different substrates. Calcium was the only metal found that protects against denaturation and also confers hydrolytic activity with these two mammalian paraoxonases.

Paraoxonase (E.C.3.1.8.1; PON1) hydrolyzes paraoxon (Aldridge, 1953a), which is an active neurotoxic insecticide, as well as disopropyl fluorophosphate, somam, sarin, and tabun. PON activity has been found in a variety of mammalian tissues, with liver and serum having the highest levels (Aldridge, 1953b), and the source of serum PON is believed to be primarily the liver (La Du, 1992). Whether the low intracellular concentrations of calcium are sufficient to fully activate PON activity in these locations has not been established. Serum PON is closely associated with apolipoprotein A-1, a major lipoprotein of the high density lipoprotein complex (Blatter et al., 1993). Human serum PON is a polymorphic enzyme, represented by A (or Q)-type and B (or R)-type isoenzymes. The B-type isozyme has a considerably higher PON specific activity, and it is stimulated to a greater degree by 1 M NaCl than the A-type isozyme (Eckerson et al., 1983). The cDNAs of rabbit and human PONs have been cloned, and the amino acid sequences have been deduced (Furlong et al., 1993; La Du et al., 1993). Two polymorphic sites are present in human serum PON: Leu/Met at position 54 and Arg/Gln at position 191 (fig. 1). The latter polymorphic site is the one responsible for the PON Q/R phenotype; Gln at position 191 determines the Q or “A” phenotype, and Arg at this position produces the R or “B” phenotype (Adkins et al., 1993; Humbert et al., 1993).

The same enzyme was proven to catalyze hydrolysis of arylesters of carboxylic acids and organophosphates (Gan et al., 1991; Sorenson et al., 1995). It is common convention, however, to use the term “paraoxonase activity” if the substrate has been paraoxon and “arylesterase activity” if phenyl acetate is the substrate. PON can also hydrolyze organophosphonates, organophosphinates, carbamates, and unsaturated aliphatic esters (La Du, 1992), so it plays an important role in the metabolism of many xenobiotic compounds.

Human serum PON requires the presence of calcium for enzymatic activity. PON activity is inhibited by metal chelating agents (e.g. EDTA or EGTA), by other metal ions, and by sulfhydryl-reagents (Erdos et al., 1960). The most potent inhibitors of PON are the rare earth metals. Some of these are active in the order of 10^-8 M, and the order of metal ion inhibitors is the following: Ce^3+ > Gd^3+ > La^3+ > Y^3+ > Sm^3+ > Th^3+ > Cd^2+ > Hg^2+ > Ag^+ > Pb^2+ > Zn^2+ > Ni^2+ > Co^2+ > Cu^2+ > Mn^2+ > Ba^2+ > Sr^2+ > Mg^2+ (Erdos et al., 1960). Erdos and Laswick (1961) found that Ca^2+ and especially La^3+ offered some protection against denaturation of arylesterase when the enzyme was treated with urea or guanidine. Marton and Kalow (1962) proposed that calcium facilitated the formation of an enzyme-substrate complex and that it accelerated the breakdown of this intermediate into enzyme and product. Zimmerman et al. (1989) found rabbit serum PON to be very dependent on calcium, and these workers maintained a concentration of 2.5 mM calcium during their purification and assays. Eckerson et al. (1982) showed a parallel dependence of arylesterase and paraoxonase activities on the calcium concentration with purified human serum esterase. Thus, calcium ion not only plays an important role in the catalytic activity of serum PON, but it also serves to stabilize the enzyme in its native molecular structure.

From our previous comparison studies (Kuo and La Du, 1995), it was determined that there are two significant differences between rabbit and human PONs: human PON is appreciably more sensitive to EDTA inhibition than rabbit PON, and Chelex 100 treatment can strip Ca^2+ more easily from the human esterase than it does from rabbit
Human Serum Low and High MW Enzymes

<table>
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<th>Species</th>
<th>Name</th>
<th>Sequence</th>
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<tr>
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<td>Rabbit</td>
<td>Y-V-T-NE</td>
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Fig. 1. Homology between human and rabbit serum PONs.

The upper line is the deduced amino acid sequence of human PON; the lower line is the sequence of rabbit PON. Dashes represent the identical amino acids with human PON; identity is about 85%. For the human species, there are two polymorphic sites that are parentheses above at positions 54 and 191. Three boxed regions represent potential calcium binding sites.

Materials and Methods

Materials. Units of human plasma were purchased from the Michigan Red Cross (Lansing, MI), and frozen rabbit sera samples were obtained from the Pel-Freez Co. (Rogers, AR). Human serum PONs were purified by three sequential gel chromatographic steps: Cibacron Blue 3GA, DEAE I, and DEAE II (Gan et al., 1991). The protocol to purify rabbit serum PON was modified; concanavalin A affinity column step (Kuo and La Du, 1995) or a Sephadex G-200 column followed the Cibacron Blue 3GA and DEAE column chromatography steps. The purified rabbit PON gave two bands on an SDS-polyacrylamide gel because of the degree of N-glycosylation. Both bands have arylesterase and paraoxonase activities (Kuo and La Du, 1995).

Removal of Contaminating Metal. Contamination was a major concern in the chemical determination of calcium at the microgram per liter level. Rigorous cleaning procedures were followed to minimize this problem. Plastic labware was used in place of glass (Powell and Tease, 1982), and soaking and deionized water were necessary (Laxen and Harrison, 1981). Disposable pipette tips and autosampler cups were also pre-rinsed with nitric acid and deionized water. Most sample handling was done in a circulation-free tissue culture hood to prevent airborne contamination. Deionized water was produced by a Milli Q system from Millipore Corp.

The buffer solution, 25 mM Tris/Base, pH 8.0, and deionized water were routinely passed through a Chelex-100 (Bio-Rad) column. The background concentration of calcium was maintained below 10^-8 M. Dialysis tubing (12,000–14,000 MWCO from Spectrum) was treated with 2% nitric acid for 2 hr and rinsed several times in deionized water before use.

Equilibrium Dialysis. Dialysis tubing samples containing 1.0 ml of 0.2–0.5 mg/ml purified enzyme in 25 mM Tris/Base, pH 8.0, 1.0 mM Ca^{2+} buffer, were dialyzed with stirring in a polypropylene bottle (Nalgene) against 1.0 liter of 25 mM Tris/Base, pH 8.0, with varying low concentrations of Ca^{2+}. Dialysis was carried out for 18–24 hr at 25°C in a circulation-free tissue culture hood until equilibrium was established.

Methods to Determine the Total and Free Concentrations of Calcium. Because a broad range of K_c values was studied for calcium binding to human and rabbit PONs, three methods were used to measure the total and free concentrations of calcium: flame atomic absorption (AA) spectroscopy (10^-4 to 10^-6 M), graphic atomic absorption spectroscopy (10^-6 to 10^-8 M), and Quin-2 fluorescence (10^-7 to 10^-8 M). A calcium reference solution, 1.0 mg/ml certified for atomic absorption spectroscopy from Fisher, was used as the common standard.

A Perkin-Elmer model 2380 single beam flame atomic absorption spectrophotometer was used to determine the total and free concentrations of calcium. The sensitivity limit of this flame AA was in the order of 10^-6 M calcium. 0.05% lanthanum (La^{3+}) solution was prepared in 3.0 M HCl as a matrix modifier. Each standard or sample was mixed with one-tenth volume of La^{3+} solution before the assay. Automatically, the machine aspirated about 0.5 ml of sample for each assay. A linear calibration curve was obtained for the range of calcium concentrations between 0.08 and 8.0 mg/liter.

For lower concentrations of calcium, we used a Perkin-Elmer model 3300 atomic absorption spectrophotometer equipped with a graphite furnace model 600 and an autosampler model AS-60. Uncoated graphite furnace tubes were used, which have lower sensitivity but a higher accuracy than pyrolytically coated ones, to determine calcium concentrations. The calibration curve was first set up for graphite tubes: step 1, temperature 120°C, ramp time 10 sec, hold time 50 sec; step 2, temperature 1100°C, ramp time 1 sec, hold time 30 sec; step 3, temperature 20°C, ramp time 1 sec, hold time 15 sec; step 4, temperature 2600°C, ramp time 0 sec, hold time 5 sec; step 5, temperature 2600°C, ramp time 1 sec, hold time 5 sec. Reading time was 2 sec at the beginning of step 4 with recording of the peak area. Each 0.9 ml of standard or sample solution was mixed with 0.1 ml of 0.2% nitric acid before each assay. Standard calibration curves were fitted to C = K_1 (K_A + K_A^3)/(K_A - 1), where C is concentration of calcium, K_1 is the observed absorbance, K_1, K_2, and K_3 are coefficients determined during the calibration procedure, and K_4 is the resolpe coefficient that is set equal to 1.0 during the initial calibration (Barnett, 1984). In applying this equation, a blank was always measured first and automatically subtracted from all subsequent measurements. All data were analyzed by a computer program, equipped with AA Lab Benchtop software. The samples were diluted sufficiently to fall in the range of the calibration curve. Each assay involved the aspiration of a 20-μl test sample solution.

The fluorescent calcium indicator, Quin-2, was employed to determine very low free calcium concentrations between 10^-5 and 10^-7 M (Dulhback et al., 1990; Linse et al., 1987; Permyakov et al., 1987). 5 mM Quin-2 from Molecular Probes, Eugene, OR was prepared in 50 mM Tris/Base, pH 8.0 buffer. The fluorescence emission of Quin-2 was excited at 340 nm and monitored at 490 nm on a PTI (Photon Technology International, Inc.) Alphascan fluorometer. Each 4-clear-sided methacrylate cuvette was washed with 2% nitric acid and then rinsed with Ca^{2+} free water. 1.0 ml of 5.0 mM Quin-2 solution and 1.0 ml of standard or sample were mixed well in a plastic cuvette and allowed to equilibrate for 5 min. The samples were excited just long enough to obtain stable readings (10 sec) because Quin-2 has a photobleaching effect after several minutes. A linear standard calibration curve was obtained between 1 and 500 nM calcium.

Protein Assay. Because of the high absorbance of tergitol (a nonionic detergent, NP-10, Sigma) at 280 nm, protein concentrations were determined by the bicinchoninic acid (BCA) protein assay (Wieschelman et al., 1988) from Pierce, Rockford, IL) with a bovine serum albumin standard.

Analysis of Binding Data. The experimental data from a Scatchard plot were analyzed with a nonlinear least squares curve-fitting program (Graph Pad-Prism, Graph Pad Software, Inc., San Diego). Four different kinetic models were used to calculate the values of association or dissociation constants of calcium for human and rabbit PONs. Analyses were done assuming that there was interaction or cooperativity between the different binding sites and also carried out assuming that each binding site was independent.
Calcium Binding by Paraoxonases

Model 1: Two independent sites–\(B_{\text{max1}}\). This model is described by eq. 1; it assumes two independent sites (no cooperativity),

\[
Y = B_{\text{max1}} X/(K_{d1} + X) + B_{\text{max2}} X/(K_{d2} + X)
\]

(1)

where \(Y\) is bound calcium; \(X\) is calcium concentration, \(B_{\text{max1}}\) and \(B_{\text{max2}}\) are the maximal binding for the first and second Ca binding sites individually, and \(K_{d1}\) and \(K_{d2}\) are the concentrations of calcium required to reach half-maximal binding for the first and second sites.

Model 2: Two independent sites. If one calcium ion is bound to the higher affinity site (\(B_{\text{max1}} = 1\)), and one calcium is bound to the lower affinity site (\(B_{\text{max2}} = 1\)), eq. 1 is simplified to eq. 2:

\[
Y = X/(K_{d1} + X) + X/(K_{d2} + X)
\]

(2)

This model also assumes that the two binding sites have no interaction.

Model 3: Stepwise equilibrium. This model assumes that the enzyme contains two distinct calcium binding sites, one with a higher affinity and the other with a lower affinity, and when the first calcium ion binds to the higher affinity site, it affects the binding of the second calcium ion (Fletcher et al., 1970). Eq. 3 for stepwise equilibrium of two binding sites model is given below,

\[
Y = (K_{f1} X^2 + 2K_{f2} K_{d1} X^3)/(1 + K_{f1} X + K_{f2} K_{d1} X^2)
\]

(3)

where \(Y\) is the molar ratio of bound ligand to macromolecule, \(X\) is free calcium concentration, and \(K_{f1}\) and \(K_{f2}\) are the first and second association constants, respectively.

Model 4: Stepwise–\(B_{\text{max}}\) equilibrium. If a new parameter called \(B_{\text{max}}\), the maximal binding coefficient, is included in eq. 3, the new equation will be:

\[
Y = B_{\text{max}} K_{f1} X^2 + 2K_{f2} K_{d1} X^3/(1 + K_{f1} X + K_{f2} K_{d1} X^2)
\]

(4)

This concept is derived from eq. 1 including \(B_{\text{max1}}\) and \(B_{\text{max2}}\) to estimate the maximal binding, and the new modified model is called the stepwise–\(B_{\text{max}}\) equilibrium model.

Arylesterase Activity with or without Calcium Assay. The ratios of Ca\(^{2+}\) bound per mole of PON were determined in the previous equilibrium dialysis experiment. To measure arylesterase activity, the assay buffer contained 1.0 mM Ca\(^{2+}\) and 1.0 mM phenyl acetate in 25 mM Tris/HCl, pH 8.0. Samples, representing different ratios of Ca/mole PON, were added to cuvettes containing the aforementioned buffer. Measurements of activity were made immediately. Assays were also conducted without added calcium in the assay buffer.

Estimation of Apparent \(K_{d2}\). A total of 0.5 g of Chelex 100 (200–400 mesh) from Bio-Rad was washed once with double distilled water and packed into a 3.0-mL polystyrene column with a plastic filter disc. The packed column was equilibrated with 50 mM Tris/HCl, pH 8.0 buffer, and then 1.0 ml of purified PON solution containing 0.5 mg protein was passed through the column at a speed of 0.3 ml/min. The fractions containing the highest enzymatic activity were pooled and saved. Various concentrations of calcium buffer were prepared (from 0 to 100 mM), and then 5-\(\mu\)l aliquots of each concentration were equilibrated with different concentrations of calcium for 30 min at 25°C to reach equilibrium. After incubation, substrate was added, and enzymatic activity was determined. Phenyl acetate, thiophenyl acetate, and paraoxon activities were determined (Augustinsson and Axenfors, 1972; Gan et al., 1991), and the apparent \(K_{d2}\) values were estimated from the concentration of calcium required to produce 50% of the maximum rate of enzymatic activity.

Results

Scatchard Plot and Hill Plot Analyses. Calcium binding by human and rabbit serum PONs was evaluated using the calcium indicator (Quin 2), graphite furnace atomic absorption, and flame atomic absorption. Fig. 2 illustrates the Scatchard plots of calcium binding by human A-type and rabbit PONs. We conclude that both \(B_{\text{max}}\) values are close to 2.0, indicating there are two calcium binding sites for human and rabbit PONs. Because both Scatchard plots were nonlinear, the two calcium binding sites can be assumed to represent different orders of affinity; one is called the higher calcium binding site, and the other is the lower affinity calcium binding site. From fig.

2, the higher calcium binding site of rabbit PON had an appreciably higher affinity for calcium than the corresponding high affinity site of human PON.

Fig. 3 shows Hill plots for the human A and rabbit PONs. The slopes (\(h\), Hill coefficient) were 0.7 and 0.4, respectively. If \(h\) value is less than 1, there are two possibilities: one is negative cooperativity, and the other is multiple nonequivalent (or independent) sites (Fersht, 1985). Thus, the two calcium binding sites in both PON species are independent. These results led us to test different molecular models to estimate the respective dissociation constants.

Analysis of the Dissociation Constants. To be consistent, all of the constants are expressed as dissociation constants. The calcium binding results (fig. 2) were analyzed using the four equations described under Materials and Methods, and the results are given in table 1. The values of \(R^2\) in two independent sites–\(B_{\text{max}}\) (eq. 1; \(R^2 = 0.967\) for rabbit, 0.998 for human) and stepwise–\(B_{\text{max}}\) equilibrium (eq. 4; \(R^2 = 0.958\) for rabbit, 0.998 for human) showed that these were the better curve-fitting models than the two independent sites (eq. 2; \(R^2 = 0.900\) for rabbit, 0.969 for human) and the stepwise equilibrium (eq. 3; \(R^2 = 0.900\) for rabbit, 0.969 for human) and that the four models were adequate representations of the experimental data. The two independent sites–\(B_{\text{max}}\) model and the stepwise–\(B_{\text{max}}\) equilibrium model both gave similar \(K_{d}\) and \(B_{\text{max}}\) values. Also, the two independent sites and the stepwise equilibrium models produced almost the same \(K_{d1}\) and \(K_{d2}\) values. Because of the large difference in the orders of affinity, all four models produce similar \(K_{d}\) values.

Fig. 4A compares the nonlinear least squares curve fitting of two independent sites with the two independent sites–\(B_{\text{max}}\) models for human A-type PON, and fig. 4B shows a comparison of the stepwise equilibrium and stepwise–\(B_{\text{max}}\) equilibrium models. A similar comparison of curve-fitting graphs of different models for rabbit PON is shown in fig. 5, A and B. From figs. 4 and 5, it is apparent that both...
two independent sites–B\text{max} and stepwise–B\text{max} equilibrium models produce the better fits than either the two independent sites model or stepwise equilibrium model.

We conclude that there are two distinct calcium binding sites with both PONs, and rabbit PON has an appreciably higher calcium binding affinity for the first calcium binding site \((K_d1)\) than human PON, but the second calcium binding sites of both species are similar.

### Calcium Binding and Enzymatic Activity

Two experimental conditions were selected to determine whether one site is essential for catalytic activity and one is responsible for structural stability. In the first experiment, the assay buffer contained 1.0 mM Ca\(^{2+}\) with 1.0 mM phenyl acetate in 25 mM Tris/HCl, pH 8.0. Specific activity was plotted against Ca bound/mol PON (bound) in fig. 6. When the ratios of bound calcium \((x)\) were greater than 1, both plots tended to reach their expected maximum specific activities (human PON A, 800 units/mg; rabbit PON, 500 units/mg). In contrast, when the bound calcium ratios were less than 1, appreciable lower specific activities were obtained. This experiment demonstrates that when bound calcium exceeded one atom per mole of enzyme, the lower affinity calcium binding site could be reactivated with additional calcium, and maximum enzymatic specific activity was recovered. However, depletion of calcium from the higher affinity site could be only partially restored in the order of 30%, by the calcium added in the assay buffer, and the loss of enzymatic activity was irreversible. In the second experiment, no calcium was provided in the assay buffer, and specific activity was again plotted against Ca bound/mole PON, as shown in fig. 6. When the ratios of bound calcium were greater than 1, the specific activities were proportional to the bound calcium ratios as indicated by the straight line. However, if the ratio of bound calcium

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**TABLE 1**

<table>
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<tr>
<th>Model</th>
<th>Human A-Type PON</th>
<th>Rabbit PON</th>
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<td>(K_{d1}) (\mu\text{M})</td>
<td>(K_{d2}) (\mu\text{M})</td>
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<td>Two independent sites–B\text{max} (^a)</td>
<td>0.670 ± 0.050</td>
<td>122 ± 43.3</td>
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<tr>
<td>Two independent sites (^b)</td>
<td>0.384 ± 0.099</td>
<td>6.22 ± 1.24</td>
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<tr>
<td>Stepwise equilibrium (^c)</td>
<td>0.362 ± 0.086</td>
<td>6.60 ± 1.20</td>
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<tr>
<td>Stepwise–B\text{max} (^d)</td>
<td>0.667 ± 0.050</td>
<td>124 ± 19.5</td>
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</table>

\(^a\) Eq. 1 was used to analyze the data. For human A PON, \(B_{max}1\) was 1.52 ± 0.04, and \(B_{max}2\) was 1.51 ± 0.23. For rabbit PON, \(B_{max}1\) was 1.32 ± 0.05, and \(B_{max}2\) was 0.69 ± 0.10.

\(^b\) Eq. 2 was used to analyze the data.

\(^c\) Eq. 3 was used to analyze the data.

\(^d\) Eq. 4 was used to analyze the data. \(B_{max}\) for human A PON was 1.52 ± 0.03, \(B_{max}\) for rabbit was 1.40 ± 0.03.
per mole of enzyme was much less than 1, the specific activities obtained were less than 10% of that expected. The latter experiment (without calcium in the assay) suggested that the lower affinity calcium binding site was reversible and required for the catalytic activity of the esterase. It should be noted that PONs were optimally supplied with calcium during the first experimental assay conditions, but the enzymes gradually lost their bound calcium during the second assay conditions and did reach an equilibrium state at the time of the assay.

Combining the results of these two experiments, we postulate that the higher affinity site ($K_{d1}$) is irreversible and essential for maintaining the stability of the enzyme, whereas the lower affinity site ($K_{d2}$) is reversible and directly involved in the catalytic activity of the enzymes.

**Experimental $K_{d2}$ Values.** To determine the calcium concentration required for enzyme activity, the following experiments were performed. A Chelex 100 column removes not only free Ca$^{2+}$ ion from the enzyme solution but also competes with calcium bound to the enzyme. It causes PON to lose activity rapidly by following this treatment. Samples of purified rabbit PON previously treated by passing it through a Chelex 100 column were treated with varying calcium concentrations and then tested for arylesterase activity as plotted in fig. 7. The curve is sigmoidal and similar to a ligand binding curve that follows the simple mass action law. Between 0 and 10 $\mu$M calcium concentrations, the arylesterase activity is dependent on the calcium concentration. At all calcium concentrations greater than 20 $\mu$M, the catalytic activity was maximal. In this way, the calculated $K_{d2}$, the concentration of calcium required to obtain half-maximal catalytic activity and presumably half-maximal binding of the lower affinity site, was experimentally determined from this plot. The $K_{d2}$ of rabbit PON was estimated to be in the order of 2 $\mu$M (fig. 7). This value is reasonably close to 5.3 $\mu$M (table 1) determined on the basis of calcium binding experiments using the stepwise equilibrium model or the two independent sites model. In contrast, the two independent sites–B$_{max}$ and stepwise–B$_{max}$ models estimated 19.0 and 115 $\mu$M, respectively. Table 2 shows that the estimated $K_{d2}$ values using three different substrates, phenyl acetate, thiophenyl acetate, and paraoxon, determined experimentally by finding the calcium concentration required to obtain 50% of the maximal catalytic activity after Chelex

![Figure 5](image_url)

**Figure 5.** Curve fitting of four different molecular models for calcium binding to rabbit serum PON.

The same comparison was made as explained for fig. 4.
column treatment of the enzymes. Rabbit, human A-type, and human B-type PON have very consistent $K_{d2}$ values of 2 to 3 μM. These results strongly support our interpretation that the lower affinity site is responsible for catalytic activity, and the higher affinity site is essential for maintaining structural stability of both PONs. It should be noted that although two independent sites–B$_{max}$ and stepwise–B$_{max}$ equilibrium models fit the experimental curve well, they produced less accurate $K_d$ values. In contrast, the two independent sites and stepwise equilibrium models produced lower $R^2$ values, but they predicted more accurately the experimentally determined values for $K_{d2}$ in the presence of substrate.

We also found that several other metal ions could preserve the human enzyme and keep it in an active form (e.g. Zn$^{2+}$, Cd$^{2+}$, Co$^{2+}$, Mn$^{2+}$, Sr$^{2+}$, Ba$^{2+}$, Mg$^{2+}$), whereas other metal ions did not do this (e.g. Hg$^{2+}$, Ag$^+$, and Cu$^{2+}$) (data not shown). The former group of metals maintains the active form of the enzyme, even though they lack any catalytic activity. The same technique was used to study Zn$^{2+}$ and Cd$^{2+}$ substituted rabbit serum PON. After purified rabbit PON was run through a Chelex 100 column, a high activity fraction was treated with an equal volume of 2.0 mM Zn$^{2+}$ or Cd$^{2+}$ in Tris/HCl, pH 8.0 buffer. Arylesterase activities were close to 0 for the Zn$^{2+}$ or Cd$^{2+}$ substituted PONs. However, after the Zn$^{2+}$ or Cd$^{2+}$ substituted human and rabbit esterases had been dialyzed against 1.0 mM Ca$^{2+}$ in Tris/HCl, pH 8.0 buffer several times, they gradually regained over 90% of their original enzymatic activity (data not shown).

From the above data, we conclude that calcium ion not only plays a role in the catalytic activity of serum PON but also serves to stabilize the enzyme in its native molecular structure. Other divalent metals such as Zn$^{2+}$ and Cd$^{2+}$ can preserve both human and rabbit PONs in their active forms, but they do not have catalytic activity with phenyl acetate and paraoxon.

**Discussion**

Previous studies have shown that rabbit PON is more stable than human PON to Chelex 100 and EDTA treatment (Kuo and La Du, 1995). The calcium binding studies reported in this paper extend these findings and offer a good explanation for these differences. The reason is that rabbit PON has 25.7 times higher binding affinity for the first Ca$^{2+}$ binding site, the one essential for enzyme stability, and thus rabbit PON is a more stable enzyme than human PON. This also explains why rabbit PON is more resistant to inhibition by calcium chelators (e.g. EDTA). However, the second calcium binding sites of human PON (6.6 ± 1.2 x 10$^{-6}$ M) and rabbit PON (5.3 ± 0.94 x 10$^{-6}$ M), required for catalytic activity, have very similar affinities. This result also agrees with previous observations that human and rabbit PONs seem to have very similar catalytic centers and also have similar substrate specificity patterns (Kuo and La Du, 1995).

The common structural feature of the calcium binding proteins is a binding site composed of a highly conserved helix-loop-helix (HLH) arrangement, about 30 amino acids in length (Kretsinger and Nockolds, 1973). Calcium is held within the 12-residue loop region of this motif, also called an EF-hand motif (Marsden et al., 1990; Strynadka and James, 1989). There are three potential calcium binding sites predicted by the EF-hand motif in the deduced amino acid sequences of human PON and rabbit PON; they are residues 77–88, 121–132, and 262–273 (fig. 1). It is well known that calcium prefers an oxygen ligand, particularly aspartic acid or glutamic acid residues. Three acidic amino acids are present in the 77–88 sequence of rabbit PON, whereas there is only one in the corresponding human PON sequence. In the 121–132 sequences, both human PON and rabbit PON have three acidic amino acids, even though the amino acid sequences are not identical. It should be noted that the 262–273 sequences of human PON and rabbit PON are identical. We have been able to demonstrate that two calcium ions are bound to human and rabbit PONs, and rabbit PON has a higher calcium binding affinity for the higher calcium binding site (for $K_{d2}$). However, the values of $K_{d2}$ of the two PONs were almost the same. The best candidate for the active center might be near in the 262–273 sequence, which is also near the one free sulfhydryl group (Cys$^{288}$). The technique of site-directed mutagenesis has been applied to our study on the active center of PON. Recombinant human A-type PON with serine in place of cysteine 283 has been constructed, and it has both PON activity and arylesterase activity with little change in the phenotyping ratio, which determines human A-type PON (Sorenson et al., 1995). This line of evidence is not in accord with Augustinsson’s proposal (1964) that a cysteine residue in PON is involved in the catalytic reaction. Other mutants are being prepared by site-directed mutagenesis to identify the location of the calcium binding sites. X-ray crystallographic analysis should not only be useful to determine the three-dimensional structure of the Ca$^{2+}$ bound form of rabbit PON.
enzyme but also to help identify the active center of PON. Calcium binding proteins that have been subjected to high-resolution crystal structure analyses fall into two general categories, according to Strynadka and James (1989). The first group includes many extracellular enzymes and proteins that have enhanced thermal stability or resistance to proteolytic degradation as a result of binding Ca$^{2+}$. For some of these enzymes, Ca$^{2+}$ may also play an additional role in the catalytic function. The second group comprises a family of intracellular proteins that reversibly binds Ca$^{2+}$ ions and thereby modulates the action of other proteins or enzymes (Strynadka and James, 1989). Serum PON is a candidate for the first type of calcium binding proteins because calcium is involved in both structural stability and catalytic activity of this esterase. In addition, the characteristics of the lower calcium binding site (K_a = 10^6 M) of PON are very similar to the calcium binding sites of calmodulin, which have the range of K_a values from 10^5 to 10^6 M, and they are reversible (Burger et al., 1984; Cox et al., 1981; Crouch and Klee, 1980; Haiech et al., 1981). The higher calcium binding site of the PONs (K_a = 10^7 to 10^10 M) is similar to the high calcium binding site family of proteins, which includes subtilisin Carlsberg, proteinase K, thermotase, and thermolysin (K_a = 10^8 to 10^10 M), in which the bound calcium is essential for maintaining the protein structural stability (McPhalen et al., 1991). So far, there is no evidence indicating that both calcium ions need to be involved in the catalytic activity of the PONs.

Ca$^{2+}$ and Mg$^{2+}$ belong to the hard metal group of ions that prefers an oxygen ligand (e.g. aspartic acid, glutamic acid) and holds the ligands by electrostatic forces (Glusker, 1991). The soft metal ions such as Cu$^+$, Ag$^+$, Hg$^+$, Hg$^{2+}$, Pd$^{2+}$, Cd$^{2+}$, and Pt$^+$ are bound to nitrogen ligands (e.g. histidine) or to sulfur ligands (e.g. cysteine) (Glusker, 1991). Fe$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Cu$^{2+}$, and Zn$^{2+}$ display intermediate properties (Glusker, 1991). We have noted that several metal ions (e.g. Zn$^{2+}$, Co$^{2+}$, Mn$^{2+}$, Sr$^{2+}$, Ba$^{2+}$, Cd$^{2+}$, and Mg$^{2+}$) can preserve the human PON activity and keep it in an active form, whereas other metal ions (e.g. Hg$^{2+}$, Ag$^+$, and Cu$^{2+}$) are unable to protect the enzyme against inactivation. The former group of metals was able to maintain the active form of the enzyme, even though it had no catalytic activity. However, Ca$^{2+}$ could displace the protective metal, and full catalytic activity could be regained.

The phosphotriesterase isolated from the soil bacteria Pseudomonas diminuta and Flavobacterium sp. has been shown to hydrolyze a wide variety of organophosphorus triesters (Donarski et al., 1989; Hoskin et al., 1995). The purified bacterial phosphotriesterase contains up to two equivalents of Zn$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Cd$^{2+}$, or Mn$^{2+}$, and full recovery of enzymatic activity could be obtained by incubation with any of these metals; Co$^{2+}$ bound enzyme had the highest specific activity (Omburo et al., 1992). In contrast, human and rabbit serum PONs require two Ca$^{2+}$ ions for enzymic stability and catalytic activity. The homology of amino acid sequences between human serum PON and bacterial phosphotriesterase is very low (less than 10%). However, the two may share some functional similarities that depend on homologous topography. Recently, the three-dimensional structure of phosphotriesterase was determined (Benning et al., 1994, 1995; Vanhooke et al., 1996), and the properties of this enzyme were also characterized. Phosphotriesterase holds two divalent metal ions surrounded by the histidine cluster and one histidine residue of the active site facilitating the reaction mechanism by general base catalysis (Banzon et al., 1995; Kuo and Rauschel, 1994; Lai et al., 1994). The specialized binuclear metal center is important to maintain its catalytic function and structural organization (Benning et al., 1994, 1995; Hong and Rauschel, 1996; Kuo and Rauschel, 1994; Lai et al., 1994; Vanhooke et al., 1996). The requirements of bacterial phosphotriesterase and mammalian PONs for two atoms of zinc or calcium suggest that both enzymatic mechanisms are metal-catalyzed hydrolyses (Lewis et al., 1988; Sorenson et al., 1995). In addition, Mn$^{2+}$ has been implicated in the organophosphatase of the squid giant axon (Hoskin, 1990).

The calcium concentration is approximately 10^{-3} M in serum, and in cells it is normally about 10^{-7} to 10^{-10} M. Therefore, serum PON should be fully active in vivo, but the intracellular PON may be only partially so. During the synthesis and processing in the liver of the PON, a glycoprotein, calcium is probably incorporated into the protein before it is secreted into the serum. The mechanism of regulation of serum PON is unknown, but the irreversible inactivation of PON by complete removal of calcium makes it a most unusual calcium binding protein. Several other metal ions that can protect human PON against irreversible inactivation (described under Results), such as zinc, have a higher affinity than calcium for human serum PON. These properties of the enzyme raise some interesting speculations of toxicological concern. Could other metals in place of calcium confer new catalytic properties to this enzyme that have not yet been identified? On the other hand, as calcium is the only metal found to date that gives PON paraoxonase and arylesterase activities, displacement by other metals with higher affinities than calcium, particularly in the tissues, might modulate and reduce the protective effect of this enzyme against organophosphate toxicity. It will be necessary to study the binding and competition with other metals in more detail to evaluate what effects other metals may have on the activity, stability, and substrate specificity of the serum and liver PONs.

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