

Stabilization of the active form(s) of human paraoxonase by human phosphate-binding protein

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Abstract

While there is a consensus that human PON1 (paraoxonase-1) has a protective role, its primary biological function remains unclear. A protective role against poisoning by organophosphates [OPs (organophosphorus compounds)] drove earlier works. Clinical interest has recently focused on a protective role of PON1 against vascular diseases. PON1 resides mainly on HDL (high-density lipoprotein) particles, and converging recent works show that both its activities and stability dramatically depend on this versatile and dynamic molecular environment. The discovery that HPBP (human phosphate-binding protein) has a firm tendency to associate with PON1 has steered new directions for characterizing PON1 functional state(s). Storage stability studies provided evidence that HPBP is involved in maintaining physiologically active PON1 conformation(s). Thermal stability studies showed that human PON1 is remarkably thermostable and that its association with HPBP strongly contributes to slowing down the denaturation rate. A hybrid PON1, displaying mutations that stabilized recombinant enzyme expressed in *Escherichia coli*, was shown to be more thermostable than natural human PON1. Predictably, its stability was unaffected by the presence of HPBP. Synergistic efforts on characterizing natural PON1 and rPON1 (recombinant PON1) provide information for the design of future stable mutants of PON1-based bioscavengers to be used as safe and effective countermeasures to challenge OPs. Maintaining a stable environment for such administrable human rPON1 should, at least, preserve the anti-atherogenic activity of the enzyme.

Introduction

Enzymes that detoxify OPs (organophosphorus compounds) are potentially useful for skin protection, prophylaxis, decontamination, treatment and remediation. Human PON1 (paraoxonase-1; EC 3.1.8.1), a calcium-dependent enzyme, was named for its ability to hydrolyse the OP paraoxon. When PON1 was found to be associated with HDL (high-density lipoprotein) particles, a new chapter in the enzyme's history was opened as it became a player in vascular physiology [1]. Although its primary function is likely to be a lactonase [2,3], PON1 displays promiscuous activities [4]: phosphotriesterase, arylesterase, lactonase and antioxidative, phospholipid-binding and anti-atherogenic properties [5,6]. Hence, certain of its alleged activities are at the moment largely debated.

The three-dimensional structure and the catalytic mechanism of human PON1 are unclear. The complete purification, an essential step for the utter characterization of a protein, is for PON1 a difficult challenge. The particular environment

of multiple interacting lipids and proteins in HDL may explain why study of PON1 in solution is difficult. Almost all of the purification procedures derive from those described in 1991 that assumed to provide PON1 at ~95% purity [7,8]. Thus abundant biochemical, biological and toxicological information have been collected for a decade, leading to partial characterization of the enzyme function [9]. Concurrently, recurrent attempts at crystallization of PON1 to solve its structure failed. Recently, the serendipitous discovery of a co-purified partner, termed HPBP (human phosphate-binding protein) [10], and the determination of the structure of a hybrid rPON1 (recombinant PON1) [11] motivated researchers to focus on understanding and overcoming difficulties to study PON1 in solution.

Being a phosphotriesterase, PON1 is regarded as a promising catalytic scavenger for the prophylaxis and therapy of OP-poisoning [12]. To acquire the status of catalytic bioscavenger, PON1 shows numerous requirements for *in vivo* safety and efficacy, of which stability issues are not the least. Indeed, thermodynamic stability is a crucial property for a prototypic OP catalytic scavenger that has to be stored for long periods of time at ambient temperatures and used under extreme climates without loss of activity. In addition, *in vivo* (functional) stability of administered PON1 controls its toxicological efficiency. Highly purified PON1 was used

Key words: catalytic scavenger, functional state, human phosphate-binding protein, paraoxonase, protein stability, purification.

Abbreviations used: CE, capillary electrophoresis; DSC, differential scanning calorimetry; HDL, high-density lipoprotein; HPBP, human phosphate-binding protein; OP, organophosphorus compound; PON1, paraoxonase-1; rPON1, recombinant PON1.

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for studying its functional stability and activity in the absence of its partner HPBP. The storage and thermal stability of the enzyme were investigated using several complementary methodological approaches. This work exemplifies enzyme stabilization through protein–protein interactions. Data obtained provide reference values for optimizing the stability of future human rPON1-based mutants with operational catalytic activity against OPs.

HPBP: partner, co-tenant or contaminant of PON1?

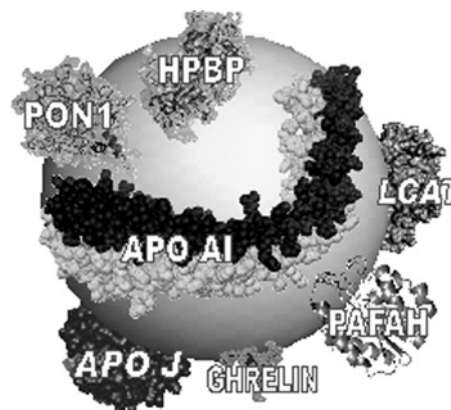
HDL offers a hydrophobic harbour for PON1 after the secretion by liver and provides a stabilizing environment for PON1 function(s) [13]. As for PON1 that displays multiple activities, HDL particles display long-lasting or transient binding capacity for numerous proteins involved in lipid metabolism, proteinase inhibitors, complement inactivation, blood coagulation, acute phase response etc. [14]. Thus HDL proteomics can be described as a pot of gold or Pandora's box [15]. Unfortunately, this huge heterogeneity is unfavourable for easily studying the function of PON1. For instance, using apparently pure human PON1 in order to solve its three-dimensional structure, we obtained crystals [16] and solved a structure not matching with the amino acid sequence of PON1. This serendipitously discovered novel protein was termed HPBP [17], and it was hypothesized to be a stabilizing partner of PON1 [18]. The unexpected presence of HPBP in allegedly pure PON1 preparations was not regarded as questionable, because a few months later several works reported the presence of various contaminants in 'pure' PON1 preparations. Several of these contaminants were found to be responsible for certain catalytic activities previously attributed to PON1 [19–21]. With recent studies highlighting the importance of the HDL-association for the biological function(s) of PON1 [13,22], converging new data indicate that both the activity and stability of PON1 are dramatically dependent on the HDL components, including HPBP (Figure 1). This protein could be involved in phosphataemia-related disorders, including atherosclerosis. Accordingly, biochemical and physiological characterization of this PON1 partner is mandatory, as is that of the environment allowing retention of the thermal and storage stability of the enzyme in solution.

Optimizing the purification process and the biochemical characterization of PON1 became priorities since the standard purification protocol yielded two proteins with similar molecular mass, PON1 and HPBP, although they have very different pI values. The harsh conditions (e.g. 9.8 M urea and 4% Triton X-100) needed to separate HPBP and PON1 by using two-dimensional SDS/PAGE indicated that HPBP is strongly associated with PON1. A two-dimensional SDS/PAGE of purified PON1 showed a major contaminant with pI value and molecular mass similar to that of HPBP, but this contaminant was not considered at the moment [23].

The PON1–HPBP interaction may well impinge directly on the function of both proteins, for example by changing

Figure 1 | Scaled schematic representation of a PON1-containing HDL particle

HDL is a ~10 nm-diameter sphere with a non-polar core of cholesteryl ester and triacylglycerol molecules encapsulated in a monolayer of amphipathic α -helical apolipoprotein and phospholipids molecules. Durably or transiently associated proteins described as having a propensity to contaminate purified PON1 fractions are shown. Unsolved three-dimensional structures (italicized) are modelled.



their specificity [24]. As a result, depiction of functions of HPBP has been actively undertaken to accumulate information on this likelihood. Because human PON1 is a promising catalytic OP-scavenger [12], it appeared essential to determine whether PON1–HPBP association is physiologically and pharmacologically relevant.

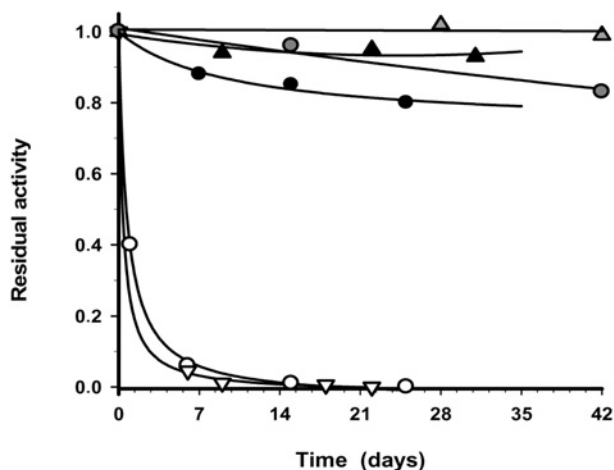
Finally, we modified the purification protocol to obtain highly purified PON1 [25]. Interestingly, this emphasized the consequence of the molecular environment for the catalytic activity(ies) and stability of the enzyme. The role of HPBP as a helper for stability and/or for OPH activity of PON1 was then hypothesized. This prompted us to investigate the balance stability/activity(ies) of PON1. Indeed, enzymes with high stability are a prime target for biotechnology, for their prolonged storage life, uses and efficiency under extreme conditions. To be a leading enzyme candidate for detoxification of OP-based agricultural insecticides and chemical warfare agents, PON1 has to be modified to improve its catalytic efficiency. Engineered novel mutants with enhanced catalytic efficiency and stability under a variety of environmental conditions implies, as a prerequisite, a careful analysis of the protein stability.

Role of HPBP in storage stability of human PON1

The changes in PON1 conformation and in its catalytic activity were monitored by using CE (capillary electrophoresis) and measurements of the activity loss of enzyme preparations stored at 4°C after the final hydroxyapatite chromatographic step. The effect of HPBP was scrutinized by performing a similar analysis on PON1 preparations

Figure 2 | Storage stability of PON1

Time course of residual activity of free human PON1 (white symbols), human PON1-HPBP complex (black symbols) and hybrid mammalian rPON1 (grey symbols) stored at 4°C. Triangles and circles indicate arylesterase and phosphotriesterase activity respectively.



supplemented with a stoichiometric concentration of HPBP immediately after purification.

CE electropherograms of free PON1 showed a decrease in the amount of its main native state and an increase in soluble aggregates. Both phenomena indicate alteration in the molecular integrity of the functional enzyme (results not shown). By contrast, in the presence of HPBP, no change in the PON1 peaks pattern was detectable after a week. This indicated that PON1 conformation was stabilized upon association with HPBP. Measurements of activity loss as a function of time of arylesterase and PON activities of preparations stored at 4°C demonstrated that both activities of free PON1 vanished after 1 week, whereas the PON1-HPBP complex retained its hydrolytic activities for several weeks (Figure 2). The results confirmed the instability of human PON1 in the absence of its physiological environment. The presence of HPBP in preparations acts as a stabilizer of the active conformation of human PON1 disencumbered of its natural HDL milieu.

A hybrid mammalian rPON1 made by directed evolution differs from the human enzyme by ~16% of amino acids. Differences located in regions involved in human PON1 binding to HDL and partner lipoproteins facilitated the expression of active and soluble rPON1 in *Escherichia coli*. Thus it seemed imperative to investigate the impact of these changes on the stability of the enzyme. Monitoring both arylesterase and phosphotriesterase activities of G3C9 rPON1 as a function of time confirmed that this variant did not lose activity for months of storage at 4°C. In addition, our attempts at supplementing rPON1 with HPBP had neither positive nor negative effect on storage stability. Electrophoretic analysis (Ferguson plot) confirmed that enzyme activity was maintained for up to 5 months. However, the remaining activity was progressively borne by formation of large aggregates with time and finally vanished. Finally, free natural human PON1

is highly unstable, whereas mammalian rPON1 is as stable as natural human PON1 complexed with HPBP.

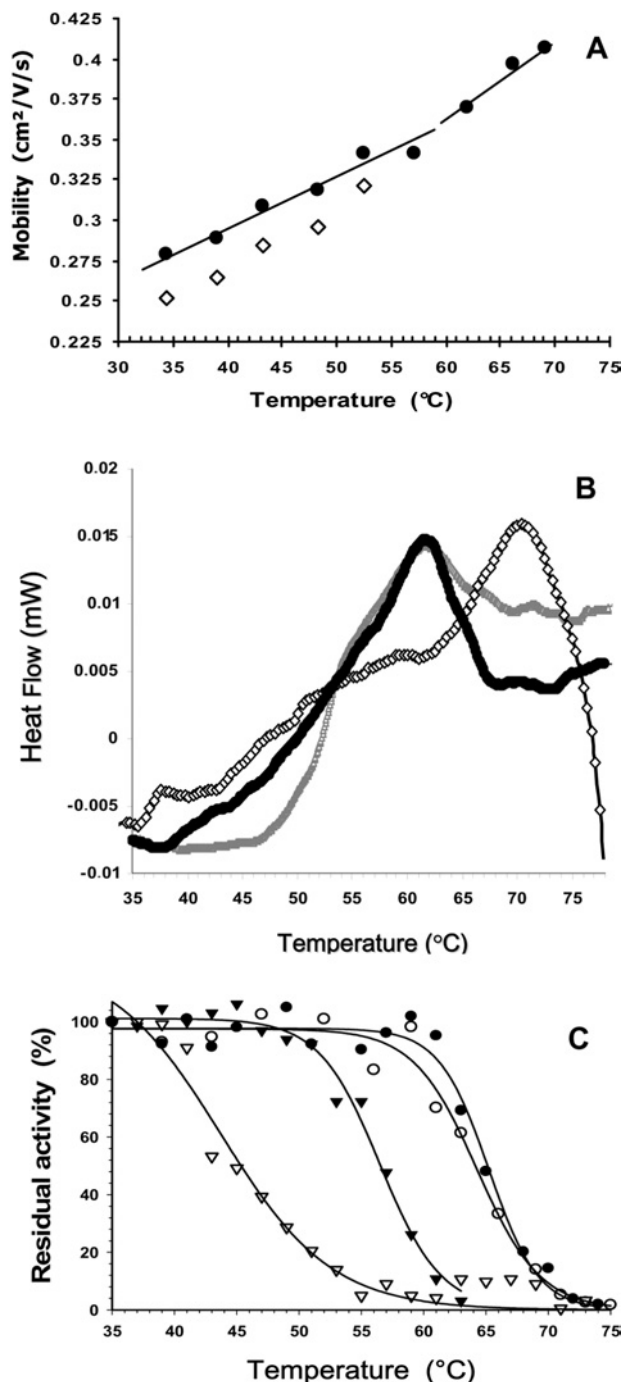
Role of HPBP in the thermal stability of human PON1

The thermal stability of PON1s was analysed using three complementary approaches: CE, DSC (differential scanning calorimetry) and thermal inactivation of its arylesterase activity (Figure 3). CE of human PON1 performed at different temperatures showed that an oligomeric form of low mobility dissociated completely above 55°C into a form of lower molecular size. Heat-induced mid-transition of this new molecular population occurred at ~60°C. Similar CE analysis of PON1-HPBP samples provided evidence for such an oligomerization change (results not shown). As shown on DSC thermograms, free PON1 displayed one main endothermic transition with $T_m \approx 61^\circ\text{C}$, preceded by an endothermic shoulder at approx. 55°C; PON1-HPBP displayed the main endothermic transition with $T_m \approx 62^\circ\text{C}$, preceded by a clearly individualized endothermic transition at 55°C. The thermal denaturation process of PON1 was shown to involve a dissociation step preceding protein unfolding, whether HPBP was present or not. However, the overall thermal stability of PON1 is high, and dissociation of oligomeric states appears to be a complex process. Measurements of residual activity after heating showed total loss of activity above 60°C for both free PON1 and PON1-HPBP preparations. Meanwhile, the presence of HPBP allowed a gain of ~12°C for the thermal inactivation mid-transition. Clearly, this indicates that thermal denaturation and inactivation of natural human PON1 are complete at 60°C. This result supports the contention that human PON1 exhibits intrinsically a remarkable thermal stability and that the association of HPBP, although it does not change the denaturation temperature, contributes effectively to slow the rate of irreversible denaturation.

The thermal stability of rPON1 was also analysed by CE, DSC and heat inactivation kinetics. CE electropherograms of rPON1 performed at different temperatures (results not shown) allowed also the detection of the two consecutive phenomena observed for human PON1, i.e. dissociation (at ~55°C) followed by irreversible denaturation. Meanwhile, the mid-transition for this thermally induced denaturation occurred at a higher temperature, 70°C. DSC thermograms of rPON1 showed a vaguely individualized first endothermic transition between 50 and 60°C, evoking the one that occurs at 55°C for human PON1-HPBP complex, followed by a main endothermic denaturation transition with a T_m of 70°C. These results demonstrated that mutations introduced by directed evolution in the rPON1 variant were not detrimental for the enzyme thermostability. On the contrary, a significant gain of ~10°C was observed. The survey of the thermal stability of rPON1, completed by studying the thermal inactivation of its arylesterase activity, provided evidence suggesting that complete loss of activity was only

Figure 3 | Thermal stability of PON1 monitored by CE, DSC and thermal inactivation

(A) The temperature dependence of human PON1 mobility from CE electropherograms at varying temperature showed that a heavy form (\diamond) dissociated completely above 55°C. For the light form (\bullet), a thermal transition between the native and the unfolded forms occurred near 60°C. (B) DSC thermograms of free human PON1 (black symbols), human PON1-HPBP complex (grey symbols) and hybrid mammalian rPON1 (white symbols) in 25 mM Tris buffer (pH 8.0) with 1 mM CaCl₂. Enzyme concentration, 1.5 mg/ml; heating rate, 0.5°C min⁻¹. (C) Thermal inactivation of arylesterase activity of human PON1 (triangles) and hybrid mammalian rPON1 (circles), free (open symbols) or supplemented with HPBP (closed symbols).



observed for temperatures higher than 70°C, allowing rPON1 to fully retain its activity at 60°C, a temperature at which human PON1 is fully inactivated.

Conclusions

Characterization of the PON1 stability/activity(ies) balance is of the utmost importance and a prerequisite for the development of variants with enhanced phosphotriesterase activity towards toxic OPs. The present review strengthens the decisive role of the environment for allowing PON1 to perform suitably and durably its catalytic activity(ies). Because PON1 exhibits an anti-atherosclerotic activity, and HPBP is assumed to be involved in phosphataemia regulation, a synergistic role of both HDL-associated lipoproteins can be reasonably argued. The key role of HPBP as a stability helper and for maintaining phosphotriesterase activity of PON1 was highlighted [26]. We established that both proteins are highly unstable in solution after dissociation. The environment allowing retaining functional enzyme state(s) and the thermal and storage stability of natural human PON1 was shown to be mandatory. The enzyme is remarkably thermostable. Its association with HPBP improves thermal and storage stability of the purified enzyme. Besides, mutations by directed evolution for soluble and active mammalian rPON1 are not detrimental for thermal and storage stability. They allow surrogating *in vitro* the stabilizing role of HPBP. This work exemplifies an enzyme stabilization through protein-protein interactions.

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