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Anuja Khan
Richard K. Hughes
Eric J. Belfield
Rod Casey
Arthur J. Rowe
Stephen E. Harding

Oligomerization of Hydroperoxide Lyase, a Novel P450 Enzyme in Plants

Anuja Khan (✉) · Arthur J. Rowe · Stephen E. Harding
National Centre for Macromolecular Hydrodynamics, University of Nottingham, School of Biosciences, Sutton Bonington LE12 5RD, UK
e-mail: sbxak2@nottingham.ac.uk

Richard K. Hughes · Eric J. Belfield · Rod Casey
John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK

Abstract The oligomeric state of fatty acid hydroperoxide lyase (HPL), of molar mass ~ 55 kDa is uncertain and it has been reported as a trimer or tetramer in vivo. The enzyme has been found to be bi-functional and is active even in the absence of detergent. The association with detergent is known to stabilise the binding of the enzyme to its substrate and the enzyme is more active. No high resolution structure of any plant P450 is available so far because of difficulty in crystallising the protein. We employ analytical ultracentrifugation to characterise the oligomeric state of an *E. coli*-expressed recombinant HPL from *Medicago truncatula* (HPL-F) under different solution conditions. Sedimentation velocity analyses show that HPL-F (under detergent-free conditions) is largely a monomer with a sedimentation coefficient $s_{20,w}$ of ~ 4.1 S (a value expected from the molar mass of the monomer). The effects of protein concentration, and detergent micelles on the oligomeric state of detergent-free HPL-F are reported for the first time. With increase in protein concentration only traces of dimers can be detected. However, HPL-F in association with detergent is a mixture of oligomers, which are not in reversible equilibrium with each other. These studies have important implications as they show that the oligomeric state of HPL-F changes with micellar association, both of which are related to the activity of the protein. They also show the virtue of combining sedimentation velocity with sedimentation equilibrium in the ultracentrifuge for the study of enzyme-detergent systems.

Keywords Analytical ultracentrifugation · Oligomerization · Oxylipin metabolism

Introduction

Fatty acid hydroperoxide lyase (HPL) is a membrane associated cytochrome P450 enzyme found in plants. It is a member of the P450 subfamily CYP74B [1]. HPL is associated with the generation of an array of oxylipins and aldehydes that play an important role in oxylipin metabolism, plant defence and is associated with developmental pathways in plants [2–4]. Some of the by-products of this pathway act as antimicrobial toxins and defend the plant against pathogen attack [5]. The C_{12} oxo-acid product of HPL is the precursor of the wound signal "traumatin" associated with wound healing in plants. The short chain aldehydes and their reduced derivative alcohols (by-products of the oxylipin pathway) are important volatile constituents responsible for the characteristic odour of fruits, vegetables, green leaves and are of immense biotechnological importance [6–8]. The enzyme cleaves the C – C bond in the hydroperoxides (HPOs), which are generated by the oxygenation of polyunsaturated fatty acids. The final by-product of the oxylipin pathway depends on where the C – C bond is cleaved in the HPO.

The study of membrane proteins is often very difficult as the protein fails to retain its "native structure" when isolated from associated membrane and this results in loss of biological activity. However the presence of detergent in solution is known to stabilise the protein structure and hence membrane proteins are usually studied in detergent which mimics the membrane environment in vivo. Because of the problems associated with isolation of such proteins in suitable form for crystallisation studies, high resolution structures are not available. The oligomeric state of HPL is uncertain and it has been reported as a trimer or tetramer in vivo in higher plants [5, 9–11]. The structure of any plant P450 cytochrome is not currently available and there is a clear need for a full biochemical and structural analysis.

The HPL under study is a recombinant enzyme from *Medicago truncatula* (HPL-F) expressed in *E. coli* at the John Innes Centre, Norwich. Studies have shown that HPL-F is a bi-functional enzyme and retains its biological activity both in the presence and absence of detergent [12]. The detergent is known to activate the protein but the mechanism by which this is done is unknown. Recent biochemical studies show that under detergent-free conditions, HPL-F has very different binding kinetics to the enzyme in buffer containing detergent and salt [12]. The difference in activity arises from the difference in conformational (oligomeric) state of HPL-F in the presence and absence of detergent. Recent studies have shown that in the absence of detergent the activity is retained but is lower than when present in association with detergent.

In this study we employ both sedimentation velocity and sedimentation equilibrium in the analytical ultracentrifuge to probe the state of oligomerisation in detergent-free and detergent containing solution conditions, thus facilitating an insight into the complex detergent-enzyme interactions in relation to functionality.

Materials and Methods

Materials

Hydroperoxide lyase (HPL-F) from *Medicago truncatula* was expressed in *E. coli* [12]. The protein was prepared in two batches. Batch one HPL-F was in 0.1 M sodium phosphate, pH 6.5, batch two was HPL-F in 50 mM potassium phosphate buffer containing 0.9 M sodium chloride, 50 mM glycine, 5% (w/v) glycerol and 0.1% (v/v) Emulphogene (polyoxyethylene 10 tridecyl ether). All chemicals for buffer preparation were supplied by Sigma Aldrich (Dorset, UK).

Sedimentation Velocity

Sedimentation analysis was employed to characterise the oligomeric state of HPL-F in detergent-free solution under the effect of increasing protein concentration, ionic strength and pH. Sedimentation velocity studies in the Beckman XL-A/I (Palo Alto, USA) were carried out on various HPL-F concentrations (0.6–15.0 mg/ml) at a rotor speed of 45–50 000 rpm, at 20 °C. The study was performed in two sets, one investigating the low concentration range (0.6–2.6 mg/ml) and the other on the high concentration range (5–15 mg/ml). Scans were recorded by interference and absorbance optics every 3.5 min. The interference scans were necessary for the high protein concentration experiments, because with absorbance optics the concentration use was limited to the Lambert-Beer range. The oligomeric state of HPL-F at concentration 0.5 and 1 mg/ml in association with detergent (emulphogene 1.6 mM) was studied using a Beckman XL-A at 45 000 rpm at 20 °C. Absorbance scans were recorded at 232 nm (to keep absorbance within Lambert-Beer range) every 3.5 min. The use of interference optics was avoided as the detergent present in association with the protein may contribute towards the signal and introduce uncertainty in the sedimentation analyses. In the present work, the concentration of emulphogene used (1.6 mM) was well above the critical micelle concentration of the detergent (0.125 mM) [13].

The raw sedimentation velocity data were analysed using the program SEDFIT [16]. The least squares $g(s)$ model within SEDFIT were employed for the analysis of the sedimentation data and for the determination of the apparent (i.e. not corrected for the effects of diffusion) sedimentation coefficient. Although the sedimentation analysis of HPL-F in detergent-free solution was relatively straightforward the analyses of the detergent-protein complexes was rather difficult due to structural heterogeneity of the protein in presence of detergent. The following procedure was therefore adopted: the $c(s)$ model was employed with the regularization [16] switched off so that the resultant "spikes" when overlayed on top of the least squares $g(s)$ distribution of the same data, greatly facilitated the identification of the peaks in the least squares $g(s)$ plot. The peaks in the $g(s)$ distribution which coincided with the $c(s)$ distribution represented the approximate sedimentation coefficient of the different detergent solubilised oligomers in solution. The proportion of each of these oligomeric forms of HPL-F was estimated by fitting multiple Gaussians via the ROBUST fitting algorithm within the software package *pro-Fit* (Quantum Soft, Switzerland) to the least squares $g(s)$ profiles.

Sedimentation Equilibrium

The weight average molecular weight (molar mass) M_w, of HPL-F under detergent-free conditions was also investigated by sedimentation equilibrium in the analytical ultracentrifuge. HPL-F samples (0.5, 1.0 mg/ml) in detergent-free buffer were run in a Beckman Optima XL-A at 20 000 rpm at 20.0 °C. Scans were taken at 280 nm

every 2 h until equilibrium was reached (after 24 h). The raw data was analysed using the MSTARA algorithm in MSTAR [14]. This procedure permits the evaluation of the apparent weight average molecular weight $M_{w,app}$ over the whole distribution of macromolecular solute in an ultracentrifuge cell (from cell meniscus to cell base), using the M^* function of Creeth and Harding [17]. Because of the low HPL-F concentrations used, it was assumed that nonideality effects were negligible and $M_{w,app} \sim M_w$.

Other Parametric Calculations

The partial specific volume, $\bar{v}$ of HPL-F (20 °C) was determined from its amino acid sequence via SEDNTERP [15]. The $\bar{v}$ of HPL-F in absence of detergent in 0.1 M sodium phosphate buffer, pH 6.5 was 0.744 ml/g. The relative viscosity and density of the detergent-buffer was measured using a viscometer and an Anton-Paar density meter respectively in order to determine the detergent contribution towards the partial specific volume of the detergent-protein complex. The measured partial specific volume for the detergent was 0.945 ml/g.

The association of the HPL-F with the detergent micelle in solution complicates the hydrodynamic interpretation of the sedimentation process and therefore it is crucial to understand how much detergent was bound per gram of the monomer protein. However for this, the partial specific volume of the protein-micelle complex has to be known. As no direct experiment was performed, and to avoid uncertainties associate with density matching (especially partial deuteration) using D_2O, a theoretical approach was employed. The method adopted for evaluation of the bound detergent is as follows:

If "x" grams of detergent is bound per gram of monomer of protein of molecular weight M_p, then the molecular weight of the monomer-micelle complex M_c is $M_p(1+x)$, The partial specific volume of complex, $\bar{v}_c$, can then be expressed as

$$\bar{v}_c = \{\bar{v}_p(1/(1+x)) + \bar{v}_d(x/1+x)\} \quad (1)$$

$$\bar{v}_c = \frac{(\bar{v}_p + x\bar{v}_d)}{1+x}, \quad (2)$$

where $\bar{v}_d$ is the partial specific volume of detergent, 0.945 ml/g. If the sedimentation coefficient of complex is s_c and sedimentation coefficient of protein is s_p, then a relation can be obtained linking the respective $\bar{v}$s and sedimentation coefficient.

$$\frac{s_c}{s_p} = \frac{M_c(1-\bar{v}_c\rho)}{M_p(1-\bar{v}_p\rho)} \frac{r_p}{r_c}, \quad (3)$$

where r_p and r_c are the radii of the monomer of protein and monomer-detergent complex.

The $s_{20,w}$ for the HPL-F monomer in association with micelle was experimentally determined in sedimentation velocity studies. Appropriate values for detergent bound to protein monomer were entered into a spreadsheet calculation and the calculated sedimentation coefficient, s_c (Eq. 3) for the complex was matched with that the experimental s-value for the monomer-micelle complex.

Results and Discussion

Oligomeric State of Detergent-Free HPL-F

The apparent distribution of the sedimentation coefficient (as obtained from SEDFIT analyses) for both low and high HPL-F concentration range show that there is only one major species in solution with weight average sedimentation coefficient of ~ 3.6 S ($s_{20,w} = 4.1$ S). An example is shown in Fig. 1, with of trace amounts of low molecular weight contaminant. The weight average sedimentation coefficient was seen to remain roughly constant over the entire concentration range studied. At high protein concentration (~ 5 mg/ml) trace amounts of a larger species of $s_{20,w} \sim 6.3$ S were detected, with a weight fraction of no more than $\sim 8.5\%$. The value of 6.3 S is consistent with a dimeric species, assuming monomer and dimer as both globular. The proportion of this species did not go up with increase in concentration indicating that there is no associative reaction within this system. A corresponding weight average molecular weight, M_w, of (55 ± 2) kDa was determined from sedimentation equilibrium studies (MSTARA analysis) on HPL-F at 1 mg/ml (Fig. 2), in excellent agreement with the sequence molecular weight for the monomer (~ 56.8 kDa).

From these observations we could conclude that the HPL-F preparation without detergent was largely mono-

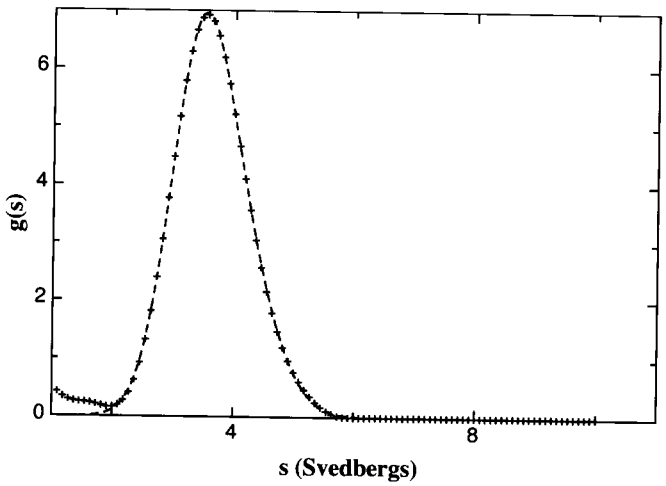

Fig. 1 Apparent distribution of the sedimentation coefficient (expressed in terms of the so-called least squares $g(s)$ distribution of Schuck [16]) for HPL-F at a rotor speed = 40000 rpm, protein concentration = 1 mg/ml. The *plusses* represent the experimental data and the *dashed line* a Gaussian fit to the main peak

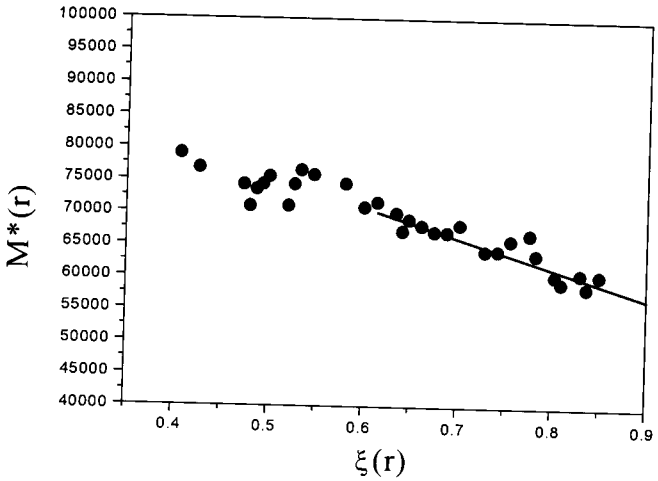

Fig. 2 Plot of $M^*(r)$ versus $\xi(r)$, where $\xi(r)$ is the normalized squared radial position. $\xi(r) = (r^2 - a^2)/(b^2 - a^2)$ where r is the radial position in an equilibrium solute distribution and a, b the corresponding radial positions at the cell meniscus and base respectively. $M^*(\xi \to 1) = M_{w,app}$, the apparent weight average molecular weight over the whole distribution in the ultracentrifuge cell (from meniscus to cell base) [17]. Extrapolated $M_{w,app} = (55 \pm 2)$ kDa. Data obtained in the Beckman-Optima XL-A at 20000 rpm, at a scanning wavelength of 280 nm, rotor temperature = 20.0 °C. Loading concentration ~ 1 mg/ml

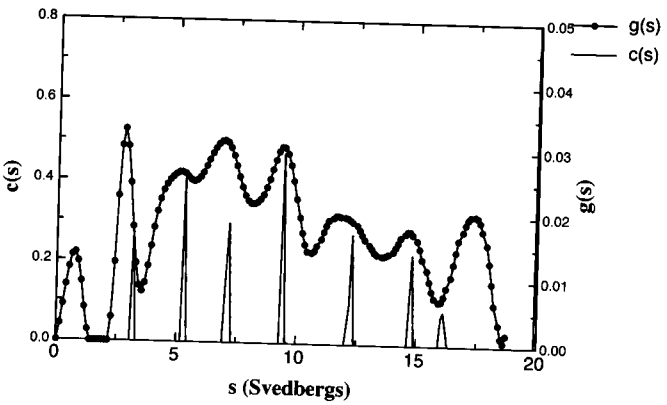

Fig. 3 Overlay of the $c(s)$ and $g(s)$ distribution data for sedimentation velocity analysis of HPL-F at a loading concentration of 1 mg/ml. The spikes represent $c(s)$ peaks used to identify the s values of each species present

meric. Kinetic data has further supported the view that the monomer was the active species in solution and was responsible for the low enzyme activity observed in the presence of the substrate [12].

Oligomeric State of HPL-F in Detergent-Buffer

Usually the sedimentation analyses of detergent-bound protein complexes are very complex and interpretation of such data is difficult. However HPL-F in association with detergent was a heterogeneous mixture of different oligomers, and the association with the micelles allowed a very distinct separation of the different oligomeric forms.

The partial specific volume of the monomer-micelle complex $\bar{v}_c$ of 0.854 ml/g was determined by applying Eqs. 1 and 2. An approximate calculation of the detergent bound (in grams) per gram of protein monomer was 1.25 (~ error ± 20%).

Analyses of sedimentation velocity data by SEDFIT have shown that detergent bound oligomers sediment at ~ 3.2, 5.3, 7.0, 9.4, 12.1, 14.8, 16.0 S (uncorrected to standard conditions) as shown in Fig. 3 for a total loading concentration of 1 mg/ml. The detergent bound to each of the oligomers will affect their buoyant density and we may have a resulting different partial specific volume for each of them: however, in our calculations we use only the average partial specific value for the monomer-micelle complex. The $c(s)$ peak at ~ 1 S corresponded to the sedimentation coefficient of the detergent micelle. It was found that decreasing the HPL-F concentration 1 mg/ml to 0.5 mg/ml revealed no net decrease in the proportion of the higher sedimentation coefficient material relative to the lower sedimentation coefficient material, an observation that was strongly indicative of a non-reversibly associative system. We estimated the proportion of each detergent associated-oligomer in solution by fitting multiple Gaussian to the least squares $g(s)$ distribution via the ROBUST fitting procedure in the spreadsheet package pro-FIT (see above) and it appears that the largest proportion of detergent associated-oligomer present in solution is a trimer. However the sedimentation coefficient of the trimer and the tetramer are very close and the association with detergent complicates the identification of the exact oligomeric form in such a heterogeneous mixture. Our finding can be related to the observation that HPL-F purified from a number of higher plants [5, 9–11] has been reported to be either trimeric or tetrameric in vivo. Even though the trimers are the most abundant species in solution whether or not they can be related to the high enzyme activity of HPL-F is yet to be ascertained.

Discussion

Sedimentation velocity and equilibrium analyses of the HPL-F in the presence and absence of detergent has allowed us to probe its oligomeric state in solution. Our findings indicate that under detergent-free conditions HPL-F was almost exclusively a monomer ($s_{20,w} = 4.1$ S) and, by contrast, a heterogenous mixture of oligomers in association with detergent. This behaviour may mimic the membrane association of the protein in vivo.

Our studies appear to have shown that the monomer of HPL-F under detergent-free conditions does not form high oligomers at high protein concentration, except for trace dimers which are not in reversible equilibrium with the

monomer. Similarly in the presence of detergent, though a number of distinct oligomers of the protein are formed they are not in reversible equilibrium with each other. The detergent micelle encapsulation of the *n*-mer (oligomer) seems to separate the oligomers quite distinctly during the sedimentation process. Our observations explain that the micelle association is favourable to the functioning of the protein as it maximises the chances of oligomer formation which can be directly related to the increased catalytic activity [12].

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