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Protein folding, unfolding and aggregation. Pressure induced intermediate states on the refolding pathway of horseradish peroxidase

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Abstract

We studied the refolding and aggregation of pressure unfolded proteins. Horseradish peroxidase was found to be very stable and no partially folded intermediates were populated during the refolding. However, the removal of the haem group or the Ca^{2+} ions or reduction of the disulfide bridge destabilized the protein, resulting in a significant amount of aggregation prone intermediate conformation. Substitution of the haem for fluorescent porphyrin however did not influence the refolding of the protein.

1. Introduction

The stability of the protein structure is determined by several factors. The main stabilizing forces are the noncovalent bonds. Hydrogen bonding stabilizes the secondary structure, but the tertiary and quaternary structures are sometimes governed by even weaker interactions. The supermolecular organization of the polypeptide chains is normally stabilized by weaker forces than the secondary structure. The role of these intermolecular interactions in formation of protein aggregation is obvious. Protein aggregates and the process of protein aggregation itself attracted considerable interest recently, because they were connected to the appearance of several neurodegenerative processes [1]. Tissue deposition of insoluble aggregates formed by proteins was shown to have an initiating role in several conformational diseases such as hereditary and acquired amyloidosis and Alzheimer disease. It is believed that the formation of highly organized amyloid aggregates is a generic property of polypeptides, and not simply a feature of the few proteins associated with certain pathological conditions [2]. These aggregates always form after some kind of destabilization of the native protein. The source of the destabilization can be mutation [3] or an environmentally induced alteration resulting in an intermediate structure with marginal stability.

High pressure has been proven to be a suitable tool for tuning protein conformations [4]. Pressure induced conformational alterations range from slight elastic deformation [5] to complete unfolding of the secondary structure [6]. Intermolecular forces seem to be even more sensitive to pressure than the ones stabilizing the intramolecular structures.

Since the aggregation is preceded by destabilization of the native structure, study of the folding of proteins is a useful approach to the aggregation phenomena. Refolding studies after chemical denaturation are well known for investigation of the folding pathway. Intermediates that are found on this pathway can play an important role in generation of aggregates. These misfolded conformations are aggregation prone.

Pressure denaturation can be reversible, unlike heat denaturation, which usually leads to a gel-like structure, trapping the protein in the unfolded conformation [7]. Pressure induced unfolding and the subsequent refolding can therefore be ideally applied for the study of protein folding–misfolding. One of the central questions of the protein folding studies is the existence of distinct intermediate states on the folding pathway [8]. Population of some of these intermediates can result in misfolding and aggregation of the protein.

It has been proven by our previous experiments that pressure treatment can populate intermediates of myoglobin. Aggregation of these intermediates was observed at slightly elevated temperature, but well below the usual heat unfolding temperature [9]. The question arises of whether the appearance of the pressure induced aggregation prone intermediates is a general feature of proteins, or whether this is only specific for certain polypeptide sequences.

There is an increasing amount of evidence showing similar behaviour in the cases of other proteins, including lysozyme and lipoxygenase. In the present study we focus on horseradish peroxidase, which is a very stable protein both against pressure and heat denaturation. We study the refolding pathway in cases of differently perturbed proteins to find intermediate structures.

2. Materials and methods

Horseradish peroxidase (HRP) was purchased from Sigma (RZ = 3.0). The purification of the basic form (isoenzyme C) to obtain a fraction with RZ = 3.4, and the substitution of the haem for metal-free mesoporphyrin (MP), are described in detail elsewhere [10].

The substrate benzohydroxamic acid (BHA), dithioerythritol (DTE) used in reduction of the enzyme and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma.

The samples for FTIR spectroscopy were prepared by dissolving the lyophilized protein in 10 mM deuterated acetate buffer (pD 5.0) at 75 mg ml^{-1}. Heavy water (D_2O) was used for all measurements in order to avoid the interference of the strong water band at 1640 cm^{-1} with the analysed amide I band. The pH meter was calibrated according to pD = pH meter reading + 0.4. The sample was stored overnight to ensure sufficient H/D exchange.

For the Ca^{2+} depleted samples EDTA was added to the protein solution in a molar ratio of EDTA:protein = 10:1.

The disulfide bonds were reduced by DTE, which was added to the protein solution a few minutes prior to filling the pressure cell. The molar ratio of DTE to protein was 100:1 in the final sample.

FTIR spectroscopy. The infrared spectra were obtained with a Bruker IFS66 FTIR spectrometer equipped with a liquid nitrogen cooled broad band MCT solid state detector. 256 interferograms were co-added after registration at a resolution of 2 cm^{-1}.

High pressure was generated in a diamond anvil cell (Diacell Products, Leicester, UK), where the pressure was built up by means of a screw mechanism. Barium sulfate was used as an internal pressure standard in all cases [11]. After the pressure cycle the sample was heated

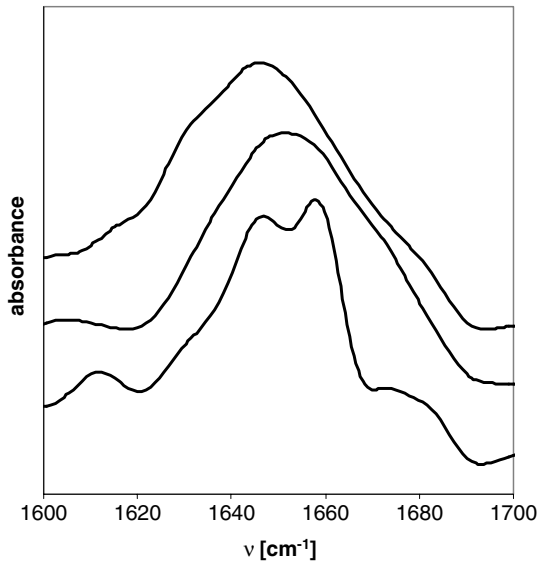

Figure 1. Amide I region of the infrared spectrum of horseradish peroxidase: the unmodified native protein at ambient temperature (bottom trace) and during (middle) and after the heat treatment of 90 °C (top spectrum).

in the DAC, by circulating thermostated water around the cell. The temperature was measured by a thermocouple positioned directly in the neighbourhood of the diamonds.

Data processing. The overlapping components of the amide I/I′ band were resolved by the Fourier self-deconvolution developed by Kauppinen *et al* [12]. The optimal parameters were determined from the observation of the power spectrum as described by Smeller *et al* [13]. A resolution enhancement factor [12] of 1.5 was reached using the Lorentzian band shape of 20 cm^{-1} bandwidth. The deconvoluted spectra were then fitted with Gaussian functions. A good fit of the amide I region was achieved by six components. The fitting of component peaks was performed by a program developed in our laboratory, using the Levenberg–Marquard algorithm [13]. The area of the fitted Gaussian line around 1616 cm^{-1} was used to characterize the intermolecular interactions.

3. Results and discussion

Unlike several proteins, horseradish peroxidase does not form intermolecular forces in the temperature unfolded state. Neither the high temperature sample, nor the cooled sample after the heat denaturation, show the specific side band which would be characteristic for the aggregation. This is shown in figure 1, where the amide I region of the infrared spectrum is shown for the native protein at ambient temperature and during and after the heat treatment.

Its known from the x-ray structure [14] and computer modelling based on these data [15] that the secondary structure of HRP is mainly helical, but a number of unordered loops are also present in the protein. Figure 2 shows the 3D structure of HRP. Several stabilizing factors can be found in HRP. First of all there are four disulfide bridges. There are also two Ca^{2+} ions, bound to the native structure of the protein. Their removal was shown to have an effect on the structure of the haem pocket. The haem itself also stabilizes the protein. All these stabilizing factors were attacked by preparing samples of HRP with reduced disulfide bridges; addition of EDTA was used to remove at least one of the Ca^{2+} ions; the haem was either substituted

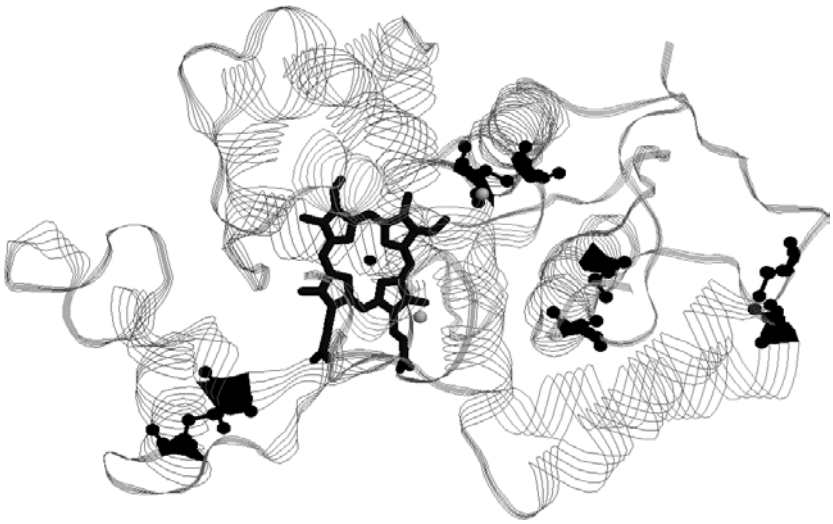

Figure 2. 3D structure of HRP. The figure was generated by Rasmol using the 1ATJ PDB file, and modifying it by selecting only one protein of the six constituting the unit cell. The grey spheres are the Ca^{2+} ions. The eight cystein residues forming four disulfide bridges are also plotted in black.

for a metal free mesoporphyrin (MPHRP), or removed completely (apoHRP). The effect of substrate (BHA) binding to the protein was also studied.

The question we addressed is whether the pressure treatment can destabilize the protein to such an extent that polypeptide chains can interact each other, forming intermolecular bounding, and finally aggregates.

Figure 3 shows the infrared spectrum of HRP after pressure unfolding and refolding, at ambient temperature and at denaturing temperature in the case of the unmodified protein and for the EDTA treated one. The lack of the side bands in the unmodified protein indicates a very strict refolding pathway, where no intermediates are formed, or these intermediates are not aggregation prone. In contrast, EDTA treatment removes at least one of the Ca^{2+} ions, and populates an intermediate state after the refolding, which can aggregate at high temperature.

Figure 4 shows the intensity of the intermolecular bands (at around 1616 cm^{-1}) of HRP variants after pressure treatment. One can see that no aggregates are formed only in the case of the substitution of the haem, or by binding a substrate. In contrast, the removal of the haem results clearly in formation of aggregates during the succeeding heat cycle. Similar results can be seen by the removal of the Ca^{2+} ions. An even more pronounced destabilization was observed in the case of reduced HRP, where the pressure unfolded protein was ready to aggregate after releasing the pressure even at room temperature.

It is well known that substitution of the haem by fluorescent porphyrins does not change the stability of the polypeptide chain. These fluorescent modifications are widely used to investigate haem proteins [10, 16, 17]. This was also supported by our previous study where we found that the pressure stability of these variants did not differ significantly from that of the unmodified protein [18].

The stabilizing role of the Ca^{2+} ion in the 3D structure was proven by NMR studies of Morishima *et al* [19]. According to them at least one Ca^{2+} is needed for the correct folding. Addition of further Ca^{2+} ions did not change the NMR spectrum [19]. Recent Raman spectroscopic measurements prove that the haem planarity was also affected by Ca^{2+} [20]. Its removal affects the nonplanarity needed for the function of the protein. In our studies the

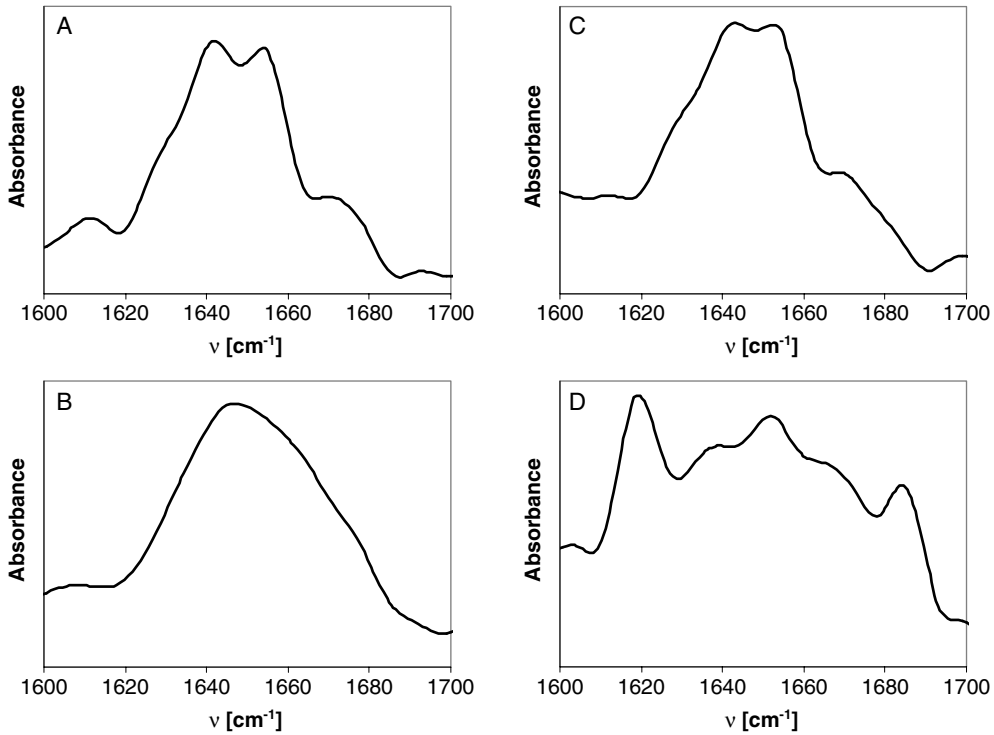

Figure 3. Infrared spectrum of native (A), (B) and reduced (C), (D) HRP after pressure unfolding (treatment up to 12 kbar) and refolding (A), (C) at ambient temperature and (B), (D) at denaturing temperature (90 °C).

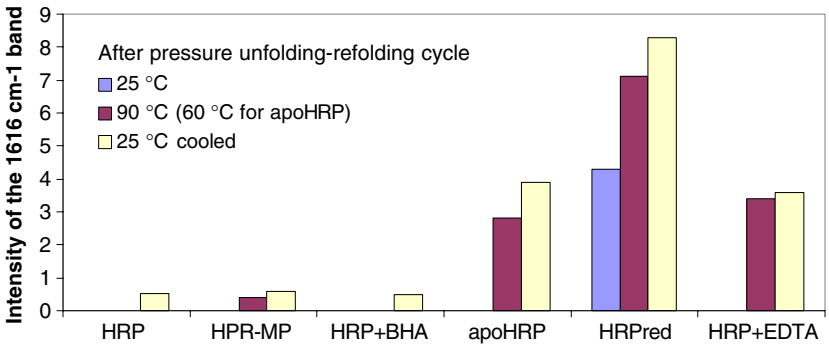

Figure 4. The intensity of the 1616 cm^{-1} infrared band representing the intermolecular interactions of HRP variants after pressure treatment at room temperature, at heat unfolding temperature and cooled back to 25 °C.

(This figure is in colour only in the electronic version)

EDTA containing sample is less pressure stable than the unchanged; however, this reduction is only ∼20% in terms of the unfolding pressure (from 12 to 10 kbar [18]). It is known from deuterium–hydrogen exchange studies that the pressure unfolded protein becomes more solvent exposed, i.e. the effect of EDTA in the pressure unfolded state can be enhanced. The

fact that a considerable amount of this protein folds into an intermediate state again underlines the structural stabilizing role of Ca^{2+}.

Reduction of disulfide bridges is clearly the most drastic change in the stability. Already the pressure stability is lowered considerably (to 8 kbar). This is the only variant where the intermediate form aggregates immediately after the release of the pressure at room temperature. In all of the other cases high temperature is needed to activate the aggregation tendency of the intermediates.

Our studies show that even in a highly stable protein such as HRP intermediate states can be generated if the protein is weakened by removing some of the stabilizing factors. Pressure unfolding–refolding is a useful tool for generating these intermediate states, which can be further studied during a heating cycle.

Acknowledgments

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