

Hemocyanin Conformational Changes Associated with SDS-induced Phenol Oxidase Activation

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Activation of Phenoloxidase by SDS

Abstract

The enzymatic activity of phenoloxidase is assayed routinely in the presence of SDS. Similar assay conditions elicit phenoloxidase activity in another type 3 copper protein, namely hemocyanin, which normally functions as an oxygen carrier. The nature of the conformational changes induced in type 3 copper proteins by the denaturant SDS is unknown. This comparative study demonstrates that arthropod hemocyanins can be converted from being an oxygen carrier to a form which exhibits phenoloxidase activity by incubation with SDS, with accompanying changes in secondary and tertiary structure. Structural characterisation, using various biophysical methods, suggests that the micellar form of SDS is required to induce optimal conformational transitions in the protein which may result in opening a channel to the di-copper centre allowing bulky phenolic substrates access to the catalytic site.

Key words: Hemocyanin, phenoloxidase, spectroscopy, enzyme activation, isothermal titration calorimetry

Abbreviations: Hc, hemocyanin; PO, phenoloxidase

Introduction

The standard assay for phenoloxidase (PO) (E.C. 1.10.3.1) activity includes the presence of low concentrations of the well-known detergent SDS (1-3). PO is present in almost all organisms, functioning as an initiator of melanin synthesis (4-7). Several potential activators of PO activity *in vivo* have been identified such as fatty acids, phospholipids, detergents, small antimicrobial peptides, and alcohols (3, 8-10). SDS, an artificial activator, seems to mimic the natural activation of PO; however, the mode of this activation is not known. It has been suggested that SDS induces a conformational transition of PO which results in an opening of the entrance to the active site (11).

It has also been demonstrated that SDS induces PO activity in the oxygen carrier, hemocyanin (Hc) (12-18). Proteolysis and modelling studies of arthropod and mollusc Hcs suggest that the natural activation of PO activity in Hcs involves the removal of an N-terminal peptide which subsequently enhances access to the di-copper centre for phenolic substrates (19, 20). This hypothesis has been supported by recent structural studies (20-24).

To date, there has been no direct biophysical characterisation of the conformational changes associated with the artificial induction of PO activity in Hcs by SDS. This study presents an analysis of the conformational changes in three Hcs associated with the addition of SDS. The Hcs investigated come from a modern chelicerate, *Pandinus imperator* and two ancient chelicerates, *Limulus polyphemus* and *Eurypelma californicum*.

Methods

Assay measurements

PO activity measurements were performed at 20 °C as described by Decker et al. (13). Typical assays included 2 mM dopamine hydrochloride plus Hc (1 mg *L. polyphemus* Hc, 0.3 mg *E. californicum* Hc or 0.16 mg *P. imperator* Hc) in 1 ml 100 mM sodium phosphate buffer, pH 7.5. Following a 5 minute incubation with SDS at a final concentration of 2.7 mM for *L. polyphemus* Hc, 5 mM for *E. californicum* Hc and 2 mM for *P. imperator* Hc. PO activity was initiated by the addition of dopamine. PO activity was followed by monitoring an increase in absorbance at 475nm resulting from the formation of dopachrome and its derivatives. One unit is defined as the formation of 1 μmol dopachrome per minute using the absorption coefficient for dopachrome of $3,600 \text{ M}^{-1}\text{cm}^{-1}$ at this wavelength. Protein concentrations were determined from UV absorbance measurements at 280 nm using the value of 1.10 for the absorbance of a 1 mg/ml solution in a cell of pathlength 1 cm for the Hc from *E. californicum* and *P. imperator* (24, 25) and a value of 1.39 for Hc from *L. polyphemus*. *L. polyphemus* Hc was purchased from A.G. Scientific and the Hcs from *E. californicum* and *P. imperator* were purified according to Decker et al. (13) and Nillius (14).

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) experiments to measure the binding of SDS to Hc were performed at 25 °C using a Microcal VP-ITC titration microcalorimeter following standard instrumental procedures (26, 27) with a 250 μl injection syringe and a stirring speed of 320 rpm. Hc was dialysed extensively against buffer (100 mM sodium phosphate, pH 7.5) and degassed gently immediately before use; SDS was dissolved in the buffer used for dialysis. A typical binding experiment involved an initial 1 μl injection, followed by 25 x 10 μl injections of SDS (30 mM, 52

mM and 75 mM SDS for *P. imperator* Hc, *L. polyphemus* Hc and *E. californicum* Hc respectively) into the ITC cell (ca. 1.4 ml active volume) containing 0.77 ± 0.015 mg/ml (10 μ M) Hc monomers (equivalent to 210 nM of the 8 x hexameric unit of *L. polyphemus* Hc and 420 nM of the 4 x hexameric unit of *P. imperator* and *E. californicum* Hc). Control experiments were performed under identical conditions (a) by injection of SDS into buffer alone to determine the CMC of SDS in 100mM sodium phosphate buffer, pH 7.5 and to correct for the heat of dilution of ligand, and (b) by injection of buffer into the protein solution to correct for the heat of dilution of the protein. Integrated heat effects, after correction for heats of dilution where necessary, were analysed by nonlinear regression in terms of a simple single-site binding model using the standard Microcal Origin software package. For each thermal titration curve, this yields estimates of the apparent number of binding sites (N) on the protein, the association constant and the enthalpy of binding. In cases of weak ligand binding the titration curve is too gradual to allow unambiguous estimation of N, and in such cases the stoichiometry may be fixed at N=1 for regression fits. Other thermodynamic quantities were calculated using standard expressions, i.e. $\Delta G^\circ = -RT \ln K = \Delta H^\circ - T\Delta S^\circ$.

Fluorescence spectroscopy

Intrinsic tryptophan fluorescence spectra were recorded at a protein concentration of 0.1 mg/ml in 100 mM sodium phosphate buffer, pH 7.5 on a Perkin Elmer LS50 spectrofluorimeter at 20 °C using cuvettes of 1 ml capacity. The excitation wavelength was 290 nm with a bandwidth of 5 nm for the excitation and emission. When fluorescence was used to monitor the effects of increasing concentrations of SDS on Hc, each spectrum was recorded when there was no further change with time; this generally required an incubation period of 5 min. All scans were recorded at a scan rate of 50 nm/min and corrected by subtraction of a spectrum of buffer alone. SDS made no contribution to the fluorescence signals under the conditions used.

Circular Dichroism

CD spectra of Hcs were recorded on either a Jasco J-600 or a Jasco J-810 spectropolarimeter at 20°C. 1S-(+)-10-camphorsulphonic acid was used to calibrate the spectropolarimeters. Spectra in the far-UV region (180-260 nm) were recorded in cylindrical cells of path length 0.02 cm, using a protein concentration of 0.3 mg/ml. Data were analysed over the wavelength range 195-240 nm with DICHROWEB, using SELCON 3 and protein reference set 3 to determine the secondary structure content. Spectra in the near-UV (260-420nm) were recorded in a rectangular cell of pathlength 0.5 cm, using a protein concentration of 0.3 mg/ml. In each case, four scans (recorded at a scan rate of 10 nm/min with a time constant of 2 s) were averaged and corrected by subtraction of a spectrum of buffer alone. Spectra recorded in the presence of increasing concentrations of SDS required a 5 min incubation with SDS prior to CD measurements for *L. polyphemus* Hc and *P. imperator* Hc, in order to allow signals to reach steady values. *E. californicum* Hc required an 16 h incubation with SDS prior to CD measurements to observe changes in the far UV CD signal.

Absorption spectroscopy

Absorption spectra of Hc samples were recorded over the range 240-380 nm. The properties of the copper binding sites were studied by monitoring the absorption peak at 330 nm, typical of type 3 copper proteins. The effects of SDS on absorption spectra were determined by incubating 0.7 mg/ml Hc with SDS for 5 min for *L. polyphemus* Hc and *P. imperator* Hc or 16 h in the case of *E. californicum* prior to absorption spectra measurements.

Dynamic Light Scattering

All dynamic light scattering measurements were recorded using a Malvern, Nano ZS (Red Badge) Differential Light Scatterer (633 nm red He-Ne laser). The Hc particle size (width and diameter) was determined in the presence of varying concentrations of SDS. 200 µl reactions containing Hc at a final concentration of 1 mg/ml, were prepared in 100 mM sodium phosphate buffer, pH 7.5, with SDS added to final concentration of 0.5 mM (submicellar) for *P. imperator* Hc and *E. californicum* Hc or SDS concentrations above the CMC (2.0 mM for *P. imperator* Hc and 5.0 mM for *E. californicum* Hc). A control solution with no SDS was prepared for each Hc type. All measurements were performed using a 100 µl DTS2145 low volume glass cuvette. Particle size measurements were recorded using 15 scans of 10 s duration, at 10 minute intervals over a total period of 40 min. A further 15 scans of 10 s duration were recorded 24 h after the initial preparation of the solutions. All measurements were recorded at 20 °C.

Dynamic light scattering was also used to confirm the CMC of SDS in 100 mM sodium phosphate buffer, pH 7.5. A series of SDS solutions (0.5 mM - 5.0 mM) were prepared in 100 mM sodium phosphate buffer, pH 7.5. Particle size measurements were recorded in a 100 µl DTS2145 low volume glass cuvette using 15 scans, each of 10 s duration at 20 °C.

Small angle X-ray scattering

Small angle X-ray (SAXS) measurements at various protein concentrations were performed in the presence of micellar concentrations of SDS. Hc from *E. californicum* was concentrated and buffer-exchanged into 100 mM Tris HCl (pH 7.8), 5 mM MgCl₂ using a Vivaspin ultracentrifugation device (Vivascience). Prior to the SAXS-measurements the samples were diluted 1:1 with the same buffer containing 10 mM SDS. The final protein concentrations used were 4.7 mg/ml, 9.4 mg/ml, and 63.5 mg/ml in 5 mM SDS.

Scattering experiments were performed using a Kratky camera with a slit collimation-system from Anton Paar (Anton Paar GmbH, Austria; type KKK). The thermostated quartz capillary containing the sample (TCS 120 holder from Anton Paar) was placed in the integrated vacuum chamber of the camera. Bremsstrahlung radiation from the X-ray generator (ISO DEBYEFLEX 3003, Rich. Seiffert & Co., Ahrensburg, Germany) was filtered using a nickel filter, beryllium windows, a wavelength sensitive counting gas (see below), and a software filter. For further treatment an effective wavelength of 0.15414 nm was assumed. The scattered intensity was recorded using a 1D position sensitive detector (PSD-50M from M. Braun GmbH, Garching, Germany) which was floated with a counting gas consisting of 90 % Argon and 10 % methane. Recorded intensities were digitized using an adapted ASA (Amplitude-Spectra Analyzer) PC board, software version 2.3 from Braun GmbH. The sample solutions were irradiated at 20 °C. Irradiation followed a pattern of 2 hours for the buffer, 4 hours for the sample, and 2 hours for the buffer again, for 30 minute intervals. Each measurement was corrected for variations of the detector efficiency, the data sets were merged and subsequently corrected for spatial distortions (28). Since SAXS measurements are very sensitive towards changes in size and shape of the sample, 30 min irradiation steps were used to minimise protein dissociation and/or denaturation. A reference data set in the same buffer including 5 mM CaCl₂ without SDS was obtained following the same irradiation procedure.

Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP- OES)

ICP-OES was used to determine the presence of copper in SDS-treated Hc using a Perkin Elmer Optima DV4300 ICP-OES instrument. Serial dilutions of copper sulphate were used to construct a standard curve. Standard solutions were prepared using 100 mM Tris HCl, pH 7.5 and subsequently diluted 5-fold in 5% nitric acid prior to analysis. Hc was incubated in the absence of SDS, submicellar concentrations of SDS or micellar concentrations of SDS for 5 min and then applied to

a NAP-5 desalting column (GE Healthcare) equilibrated with no SDS, submicellar concentrations of SDS or micellar concentrations of SDS, respectively. Protein was detected in the NAP-5 eluant spectrophotometrically at 280 nm. Copper ions were detected using ICP-OES at wavelengths of 327.373 nm and 324.749 nm (wavelengths characteristic of copper emission with minimal interference from contaminants). Fractions from the NAP-5 column were diluted 5-fold in 5% nitric acid prior to ICP-OES analysis. All ICP-OES experiments were carried out using 100 mM Tris HCl, pH 7.5 rather than 100 mM sodium phosphate buffer, pH 7.5 due to problems associated with the insolubility of copper phosphate.

Results

Phenoloxidase activation by SDS

The SDS-induced PO activity of Hc was determined by measuring the rate of formation of dopachrome and its derivatives (Figure 1). Control measurements showed that SDS did not stimulate the formation of dopachrome and its derivatives from dopamine in the absence of Hc. The CMC for SDS under the assay conditions was 1.05mM (as determined by isothermal titration calorimetry (Figure 2) and dynamic light scattering measurements (Figure 3 (a))). The PO activity of Hc was measured in the presence of 0 - 1.05 mM SDS, conditions favouring the presence of SDS monomers. Activity data were also recorded in the presence of 1.05 – 40 mM SDS, conditions under which the micellar form of SDS predominates. Figure 1 shows that monomeric SDS, at a concentration lower than 0.75 mM, fails to induce significant PO activity. However, at higher concentrations of monomeric SDS (0.75 to 1.05mM) and in the presence of SDS micelles (>1.05 mM SDS), Hc exhibits PO activity. Optimal activities of 2.8 units/mg, 12.8 units /mg and 60.5 units/mg for *L. polyphemus*, *E. californicum* and *P. imperator* Hcs, respectively, required micellar

concentrations of SDS. At SDS concentrations above 10 mM, the PO activity decreases markedly for *L. polyphemus* and *P. imperator* Hcs. The biphasic nature of this response curve is indicative of enzyme-micelle interactions (29). The SDS-induced PO activity of *E. californicum* Hc appears to be more stable with concentrations of 10-40 mM SDS causing only a modest (30%) decrease in the PO activity. A number of biophysical techniques were employed to characterise the nature of conformational changes in Hc associated with the SDS-induced stimulation of PO activity.

Isothermal Titration Calorimetry

Control titration experiments with SDS (in the absence of Hc) were used to determine the CMC of SDS, see upper panels in Figure 2. Endothermic signals arising from the dissociation of micelles to monomers, upon dilution of the SDS from the injector into the cell, ceased once the SDS concentration in the cell reached CMC.

The initial steps of titration of Hc with SDS produced an endothermic response due to micelle dissociation, in a manner similar to the control titration experiments. Increasing SDS concentrations produced a biphasic exothermic response in *L. polyphemus* and *P. imperator* Hc (Figure 2 (a) and (b), respectively), which may be indicative of an initial binding of monomeric SDS molecules to Hc, followed by interactions with SDS micelles. The heat changes during the titration of *E. californicum* Hc with SDS were negligible ($< 1.0 \mu\text{cal}/\text{sec}$, data not shown) due to the lengthy pre-incubation time required to induce conformational changes in this stable protein. The resistance of *E. californicum* Hc to SDS binding and associated denaturation is highlighted by the dynamic light scattering results in Figure 3 (b).

Effect of SDS on secondary structure

The far UV CD spectra revealed a small but significant change in the secondary structure content of all three Hcs when incubated with SDS (Figure 4). Although sub-micellar concentrations of SDS induced a change in the secondary structure, larger changes were observed at the CMC of SDS. When the data were analysed over the wavelength range 195-240 nm with DICHROWEB, it was concluded that the α -helical content increased, the β -sheet content decreased whilst the turn and unordered structure contents remained essentially unchanged for all three Hcs (Table 1). It is worth noting that in the case of *E. californicum* Hc, which required a 16 hour incubation in 5mM SDS to elicit these small but significant changes in secondary structure, denaturation was not observed. Similar lengthy incubations with micellar concentrations of SDS led to the loss of the far UV CD signal (data not shown) for Hc from *L. polyphemus* and *P. imperator*, indicating denaturation had occurred.

Effect of SDS on tertiary structure

SDS-induced changes in the tertiary structure of Hc were characterised by recording intrinsic fluorescence intensities and near-UV CD spectra. The different Hcs contain between 5 and 8 tryptophan residues per subunit. Increasing SDS concentrations resulted in a small decrease (2-5 nm) in the wavelength of fluorescence emission maximum for *L. polyphemus* and *P. imperator* Hc suggesting that some or all of the tryptophan residues become less exposed to the solvent (Figure 5 (a) and (b)). The presence of SDS also led to a marked increase (some 4-fold) in the intensity of fluorescence for Hc from *L. polyphemus* and *P. imperator* which is indicative of a reduction of internal quenching. A similar increase in intensity of fluorescence (though accompanied by a small (5 nm) red shift in the emission maximum) was observed in *E. californicum* Hc Figure 5 (c); however a 16 hour incubation with 5 mM SDS was required to elicit this response.

Addition of sub-micellar concentrations of SDS resulted in some change in the Hc emission spectra, however, concentrations of SDS approaching the CMC (0.8 - 1.05 mM) and above this value, produced the maximum change. This suggests that SDS concentrations approaching and exceeding the CMC are required to induce the more marked conformational change resulting in some tryptophan residues moving to an environment in which less fluorescence quenching occurred.

A control experiment in which SDS was added to the model compound N-acetyl-L-tryptophanamide indicated that the SDS-induced changes in Hc fluorescence were not the result of a direct interaction between the detergent and exposed tryptophan side chains in the protein, see Figure 5 (d).

Dynamic light scattering measurements suggest that the presence of SDS has no effect on *P. imperator* and *E. californicum* Hc particle size over the timescale required to observe changes in activity and in secondary structure. *P. imperator* Hc is stable over 40 minutes in the presence of micellar (5mM) and sub-micellar (0.5mM) concentrations of SDS, while *E. californicum* Hc appears to be extremely stable, with little structural change over 1440 minutes under similar conditions. The stability of *E. californicum* Hc in SDS permitted the study of conformational changes in this protein by SAXS. As can be seen in Figure 6, the pronounced peaks of the distance distribution function diminish while the protein apparently “shrinks” upon addition of SDS. This can be explained by a more parallel orientation of two pairs of hexamers of *E. californicum* Hc, in a manner similar to the conformational changes observed upon deoxygenation (30). Both dynamic light scattering and SAXS results indicate that the observed changes in fluorescence are not due to changes in the degree of exposure of tryptophan residues located at subunit interfaces.

The intensity of the near-UV CD spectral peaks (in the 260 – 320 nm range) of all three Hcs was found to decrease substantially (by 30% at micellar concentrations of SDS and by 70% at 3.5 mM SDS) following incubation with increasing concentrations of SDS (Figure 7), consistent with a

substantial change in the environment of the aromatic residues. The changes were greatest at SDS concentrations approaching the CMC (0.8mM -1.05 mM) or higher. The spectral changes in the range from 260 to 320 nm are consistent with conformational changes that alter the environment around at least some of the aromatic residues in the proteins.

Thus intrinsic fluorescence intensities and near-UV CD spectra both indicate significant structural changes in Hc upon the addition of SDS at concentrations close to the CMC and this process coincides with the induction of PO activity in Hc.

Presence of Protein-bound Copper (CuII)

Hcs and POs with bound dioxygen exhibit unique absorption spectra with a characteristic absorption peak at ~350 nm with an $\epsilon \sim 20,000 \text{ M}^{-1}\text{cm}^{-1}$ (31). This absorption band results from the peroxide which is present in a $\mu\text{-}\eta^2\text{:}\eta^2$ side-on arrangement and acts as a strong σ -donor ligand to the Cu(II) ions. Addition of SDS results in a reduction in the intensity (Figure 8) of this absorption band in all three Hcs. The di-copper centre of Hcs is also responsible for the characteristic near UV CD negative signal at ~340 nm (Figure 7) (32). While ICP-OES measurements clearly indicate the presence of two copper ions per Hc subunit following treatment with micellar concentrations of SDS (see Table 2), more detailed spectroscopic and structural studies will be required to establish the molecular nature of the protein-bound copper in Hc under these conditions.

Discussion

Hemocyanin and phenoloxidase (E.C. 1.10.3.1) are members of the type 3 copper proteins, containing binuclear copper centres (31, 33, 34). Despite the close relationship between the two proteins, Hc functions as an oxygen transporter in many chelicerates and arthropods, whereas PO initiates the synthesis of melanin and is involved in the immune response, wound healing, browning and the sclerotisation process in arthropods after molting. Several studies have indicated that it is possible to convert oxygen-binding Hc to a functionally active PO (5, 13, 17, 19 and 35). Limited proteolysis of *E. californicum* Hc results in the release of an N-terminal fragment with the concomitant development of PO activity, an observation which may reflect a dual role for Hc *in vivo* (19). PO activity can also be elicited in both PO and Hc by incubation with SDS (12-18). SDS is an artificial activator and is used routinely in PO assays for detection of PO activity, both in standard assays (1-3) and in activity gels (36). It is assumed that the presence of SDS mimics the effects of natural activators (37) (such as fatty acids, phospholipids and small antimicrobial peptides) and interacts with PO and Hc without inducing denaturation.

Hcs in this study are the 4x6-mers from the tarantula *Eurypelma californicum* and the scorpion *Pandinus imperator* and the 8x6-mer from the horseshoe crab *Limulus polyphemus* (38, 39). All three organisms belong to the chelicerate subphylum of the arthropods. Recent studies of chelicerates suggest these animals lack the PO protein and that any required PO activity is performed by Hc. This would involve the conversion of Hc from an oxygen carrier to an enzymatic PO, via an unknown structural change (6, 40, 41). In the case of arthropods in which the protein PO has been found to occur, PO appears to be closely related to arthropod Hc with respect to sequence (>30% identity) and 3-dimensional structure (41-44). Indeed, PO from the crustaceans *Astacus leptodactylus* and *Palinurus elephas* were found to be structurally similar to the arthropod hexameric Hcs (42). Thus, arthropod PO and Hc are very similar, suggesting that they would have similar stability and activation properties.

Arthropod Hc subunits (molecular mass ~72 kDa) are folded into three domains: the N-terminal domain I consists of α -helices, domain II contains a four- α -helix bundle which carries the di-copper centre and domain III consists of a seven stranded β -barrel. Three kidney shaped subunits associate via their concave surfaces to form a planar trimer. Two trimers dimerise, sandwich-like, after a rotation of about 60° against each other to form a hexamer (38). These hexamers are structurally tightly interconnected and are thermostable at temperatures up to 80°C (45). The Hc from *E. californicum* retains its structural integrity at high temperatures (T_m ~91 °C), and in the presence of denaturing agents and proteases (46).

Oxygen binding by multi-hexameric Hcs (1x6, 2x6, 4x6, 6x6, 8x6, depending on species) can be described according to the classical Monod, Wyman and Changeux model of allosteric proteins (47-50). Hexamers or dodecamers of Hc are coupled allosteric units which undergo a conformational transition. Oxygenation of Hc promotes the formation of different conformational states, involving all Hc subunits (30, 51-54). Structural studies of the oxy- and deoxy-*L.polyphemus* subunit II Hc suggest that transition from the low oxygen affinity state to the high oxygen affinity state is accompanied by domain I twisting against domains II and III in all six subunits simultaneously, which results in a withdrawal of Phe 49 and an opening of the entrance to the di-copper centre (55, 56). Phe 49 is highly conserved among arthropod Hcs and the structurally similar arthropod POs. It has been proposed that activation of Hcs to exhibit PO activity, and to activate POs, requires complete removal of Phe 49 to open the entrance to the di-copper centre and make the active site accessible to bulky monophenols and diphenols (20, 23).

Our findings prove clearly that Hc from the ancient chelicerates *L. polyphemus* and *E. californicum*, and from the modern chelicerate, *P. imperator* undergo a conformational change upon the SDS-induction of PO activity. Previous inhibitor binding (57) and molecular modelling studies (20) suggest that the di-copper centre of PO is more accessible than the oxygen binding site of the

closely related oxygen carrier, Hc. This biophysical study of the SDS- induction of PO activity in Hc suggests that there is enhanced substrate access to the di-copper centre at SDS concentrations approaching the CMC. This is supported by activity measurements which coincide with conformational changes which have given rise to changes in CD and fluorescence signals. The increase in SDS-induced PO activity at CMC (1.05mM) coincides with an increase in the secondary structure of Hc (Figure 9). Tertiary structure changes precede secondary structure changes, at SDS concentrations approaching the CMC (0.85-1.05mM). Although this suggests an initial increase in flexibility, possibly induced by monomeric SDS, it is the micellar form of SDS which promotes the optimal conformational changes required to elicit maximal PO activity. Such changes are characteristic of the formation of a 'molten globule' state (58) and may represent a functionally important state (59) of Hc from chelicerates, which lack the enzyme PO. Our SAXS results exclude the possibility that SDS induces swelling of Hc as the result of some unfolding and/or dissociation process. Recent studies on SDS-activation of field bean polyphenol oxidase (60) resulted in a substantial Stokes radius increase (from 49.1 ± 2 to 75.9 ± 0.6 Å) suggesting unfolding accompanied the activation of this enzyme. In the case of chelicerate Hc, the quaternary structure remains intact, the protein adopts a near native conformation and the conformational change 'opens the path' for potential substrates.

Hc from the modern chelicerate *P. imperator* exhibited the highest specific activity for SDS-induced PO activity; however, its quaternary structure was compromised following prolonged (over 40 minutes) incubation with submicellar and micellar concentrations SDS, indicating more flexible or open access to the di-copper centre. Hc from the ancient chelicerates, *E. californicum* and *L. polyphemus*, exhibited lower PO activity suggesting more restricted substrate access to the di-copper centre. *E. californicum* Hc appeared to be the most stable structurally of all three Hcs in this study, with higher concentrations of SDS required to observe activation and longer incubations in

the presence of these higher concentrations of SDS required to observe structural changes. In addition, there was only a relatively small decrease in PO activity following prolonged incubation in the presence of SDS and no apparent change in the quaternary structure following extensive incubation with micellar and submicellar concentrations of SDS. Thus it would appear that this ancient Hc has low levels of stable SDS-induced PO activity whereas the modern Hc has evolved to exhibit a higher PO specific activity and reduced stability. Under mesophilic conditions, lower levels of activity and enhanced structural stability are properties which have been observed in thermophilic enzymes relative to their mesophilic counterparts (61, 62). The reduced specific activity and enhanced stability are associated with reduced conformational flexibility. At higher temperatures, similar to the optimal growth temperatures of thermophilic organisms, thermophilic enzymes exhibit specific activities similar to their mesophilic counterparts at mesophilic growth temperatures. In the case of Hc from *E. californicum*, an organism which tolerates temperature fluxes of 70°C, this protein remains stable and functional as an oxygen carrier at temperatures up to 90°C (46). The high resistance to prolonged incubation with micellar concentrations of SDS may reflect a conformational transition which mimics the normal flexibility of *E. californicum* Hc at higher temperatures, allowing the protein to remain folded and fully functional. This flexible conformation may also promote the access to the di-copper centre allowing bulky phenolic PO substrates into the catalytic site. While Hc from *L. polyphemus* and *P. imperator* are not as stable as *E. californicum* Hc, they do share a similar conformational change in the presence of micellar concentrations of SDS which also promotes PO activity. The natural induction of PO activity in Hc will require further investigation. It is anticipated that the conformational changes induced by the natural activation of PO activity in Hc will mirror the changes observed in this study.

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Figure 1: Activity vs SDS (V/VO vs [SDS])

Figure 2: ITC data (a) determination of CMC (b) Hc binding SDS

Figure 3: Dynamic light scattering

Figure 4: Far UV CD

Figure 5: Fluorescence including control

Figure 6: SAX

Figure 7: Near UV CD

Figure 8: Absorbance at 350nm

Figure 9: Correlation of far UV

Table 1: Predicted secondary structure

Table 2: ICP OES

Table 3 (P(r)).

Key words: Hc, phenoloxidase, spectroscopy, enzyme activation, isothermal titration calorimetry

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