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SDS-induced phenoloxidase activity of *Helix aspersa* maxima hemocyanin

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Dedicated to Acad. Dimiter Ivanov on the occasion of his 120th birth anniversary

Oxygen-transporting protein of the hemolimph of snails *Helix aspersa* maxima (HaH) was converted from being an oxygen carrier to a form which exhibited phenoloxidase activity by incubation with sodium dodecyl sulfate (SDS). On treatment with 1.73 mM SDS, for 3 min, significant increase of catalytic efficiency of hemocyanin towards substrate catechol (k_{cat}/K_m = 34.56 mM.min^{-1} versus k_{cat}/K_m = 0.093 mM.min^{-1} for native hemocyanin) was achieved. The highest *o*-diPO activity and enzyme efficiency of HaH (k_{cat}/K_m = 60.94 mM^{-1} min^{-1}), after incubation in 1.73 mM SDS for 3 min, was determined towards substrate dopamine. Structural characterization by means of absorption and fluorescence spectroscopy and circular dichroism showed that SDS induced optimal conformational changes in the protein. As a result the active sites become more accessible to the molecules of the substrate and the hemocyanin can function as phenoloxidase.

Key words: hemocyanin; *Helix aspersa* maxima; phenoloxidase activity; enzyme activation

INTRODUCTION

Hemocyanins (Hcs) are high molecular mass copper proteins, freely dissolved in the hemolymph of invertebrates from the phyla *Mollusca* and *Arthropoda* [1,2]. Molecules of molluscan Hcs are structured as decamers (cephalopods) or didecamers (gastropods) of subunits with molecular mass of 350-450 kDa [3]. The subunit polypeptide chains are organized as a linear sequence of seven or eight globular functional units (FUs) of ~ 50 kDa, each containing a binuclear copper active site capable of reversibly binding dioxygen. The binuclear copper active site has two closely spaced copper atoms each coordinated by 3 histidine nitrogen atoms. Dioxygen is reversibly bound to the active site as peroxide in the characteristic side-on bridging (μ-η^2:η^2) geometry (Fig. 1) [4-7]. Similar active site geometry possess the other members of the type-3 copper protein family enzymes i.e. tyrosinase (Ty) and catechol oxidase (CO). Although the binuclear site is highly conserved, as witnessed by its characteristic spectroscopic properties and sequence homology, the functionality of these proteins is different. Namely, Ty catalyzes both the *o*-hydroxylation of monophenols to *o*-diphenols (tyrosinase or monophenoloxidase activity) and subsequent oxidation of *o*-diphenols to *o*-quinones (catecholase or diphenoloxidase activity), whereas CO catalyzes only the second reaction (Fig. 2) [7].

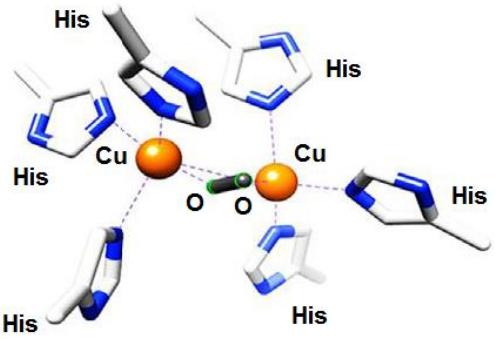

Fig. 1. Binuclear copper active site with six conserved histidine residues, indicative of type-3 copper proteins.

In contrast to these dicopper enzymes, the function of Hcs is restricted to reversible binding of O_2. The inaccessibility of the type-3 center to potential substrates has been accepted as one of the main reasons for the absence or low level of phenoloxidase (PO) activity in Hcs [8]. After activation of Hcs, making the active sites more accessible to the molecules of the substrate, they can function as POs [9]. Thus, upon treatment of

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the Hc, isolated from snails *Helix aspersa* maxima (gastropod) with proteolytic enzyme, more than sixty fold increase in its enzymatic efficiency was achieved [10].

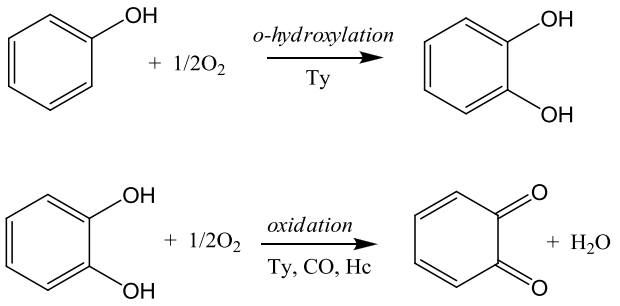


Fig. 2. Hydroxylation step (top) and subsequent oxidation (bottom) mediated by tyrosinase (Ty). The later reaction is also catalyzed by catechol oxidase (CO) and hemocyanin (Hc).

This study presents the SDS-induced phenoloxidase activity of *Helix aspersa* maxima hemocyanin (HaH), as well as an analysis of the accompanying conformational changes in the protein, which gives further insight for the enzymatic properties of these multifunctional proteins.

EXPERIMENTAL

Reagents

L-Dopa and dopamine hydrochloride were purchased from Sigma-Aldrich. Catechol was obtained from Merck. All other chemicals used are of analytical grade.

HaH was isolated from the hemolymph of the snails *Helix aspersa* maxima and purified as described in [10].

Assay of phenoloxidase activity

In the standard procedure, the PO activity of HaH was determined at 25°C in 25 mM sodium phosphate buffer, pH 7.2, at a final concentration of Hc of 0.5 mg/ml. The enzyme reaction was followed by absorbance measurement of quinone derivatives, formed as a result of substrate oxidation at 400 nm (for substrate catechol) or 475 nm (for substrates L-Dopa and dopamine). The absorbance values were corrected for the contribution due to the autooxidation of the substrates by running the same assay in the absence of Hc. The reaction rate was measured from the initial quasi-linear portion of the curves (usually 0-3 min). The kinetic parameters [K_m (mM), V_{max} (nmol min^{-1} mg^{-1}) and k_{cat} (min^{-1}), expressed per copper pair] were derived from non-linear regression data analysis of the dependence of the initial rates on the substrate concentration (according to Michaelis-Menten kinetics) using HYPER software (Hyperbolic Regression Analysis of Enzyme Kinetic Data, Copyright J. S. Easterby) [11], taking into account the molar absorption coefficient of 1417 $M^{-1}cm^{-1}$ for *o*-quinone [12] and 3600 $M^{-1}cm^{-1}$ for dopachrome [13], and the molecular mass of a FU (entity with one active site; 50 kDa). The kinetic parameters were determined from three experiments and reported as mean ± standard deviation.

UV-VIS absorption spectroscopy

UV-VIS spectrophotometer model Evolution 300, Thermo Electron Corporation, equipped with a Peltier temperature control accessory, was used for the kinetic measurements. Specific absorption coefficient $a_{278\ nm}$ = 1.413 ml mg^{-1} cm^{-1} for HaH [14] was used for determining the protein concentration.

Steady-state fluorescence measurements

The fluorescence spectra of HaH were recorded by means of a spectrofluorimeter Perkin Elmer model LS55, at an excitation wavelength of 295 nm. In order to avoid inner filter effects, the optical density of protein samples was < 0.05.

Circular dichroism

Far-UV circular dichroism (CD) measurements were performed at 25°C with a Jobin-Yvon CD6 spectropolarimeter in the 190-250 nm region, using a cell with 0.1 cm pathlength. The protein was dissolved in 25 mM phosphate buffer, pH 7.2, at a concentration of 0.1 mg/ml. Spectra in the near-UV (260-420 nm) were recorded in a rectangular cell of pathlength 0.5 cm, using a protein concentration of 0.3 mg/ml. In each case, five scans were averaged and corrected by subtraction of a spectrum of buffer alone.

RESULTS AND DISCUSSION

Phenoloxidase activation by SDS

Recently, we have established that the native HaH is able to catalyze the reaction of oxidation of catechol to *o*-quinones, i.e. possesses *o*-diPO [10]. The V_{max} value for HaH was obtained to be 22 ± 0.001 nmol min^{-1} mg^{-1}, corresponding with a k_{cat} = 1.1 min^{-1} (expressed per dinuclear copper active site) (Table 1). The native HaH did not show any *o*-

diPO activity with L-Dopa and dopamine as substrates. Similar results have been reported for other molluscan Hcs, namely Hcs isolated from terrestrial snail *Helix pomatia* (β-HpH) and marine snail *Rapana thomasiana* (RtH) [15,16]. Sodium dodecyl sulphate (SDS) is effective in activating Hcs. It has been shown that the micellar form of SDS (present at concentrations > 1 mM in 100 mM sodium phosphate buffer, pH 7.5) is required to induce optimal conformational transitions in the protein, which may result in opening a channel to the di-copper center allowing bulky phenolic substrates to access the catalytic site [17]. Different concentrations of the detergent SDS (0.35-3.81 mM; final concentrations) and incubation times were used in our study to induce *o*-diPO activity in HaH. High degree of increase of the *o*-diPO activity of HaH (v_i ~ 0.75 μmol min^{-1} mg^{-1}) towards substrate catechol, was achieved after 3 min incubation in SDS at concentrations from 1.04 mM to 2.08 mM (Fig. 3).

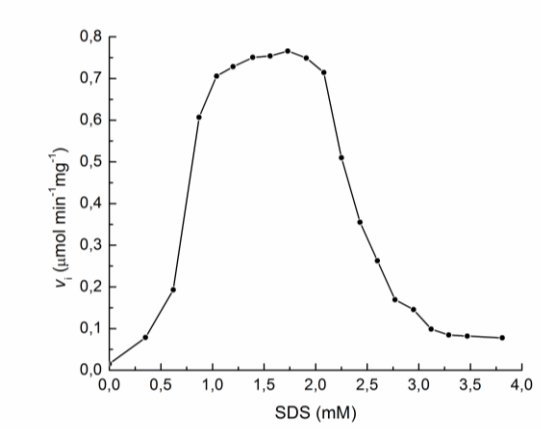


Fig. 3. *o*-diPO activity of HaH, after 3 min incubation with SDS (0.35-3.81 mM; final concentrations). The assay was carried out with catechol (1 mM) as substrate in 25 mM phosphate buffer, pH 7.2, at 25°C and Hc concentration of 0.5 mg/ml.

Higher concentrations of SDS (>2.08 mM SDS) most probably affect the catalytically active conformation of the binuclear copper active sites, thus the induced activity again decreases (Fig. 3). Conformational changes in the protein associated with the addition of SDS have been analyzed by various biophysical methods.

Effect of SDS on the secondary structure of HaH

The far-UV CD spectrum of native HaH shows two distinct negative Cotton effects at about 208 nm and 222 nm due to the the α-helix and β-sheet structures of the protein. CD spectra taken after 3 min of incubation with different concentrations SDS (1.2-2.6 mM) for 3 min at 25°C show changes in the secondary structure, as indicated by the shift of the minimum at 208 nm to lower wavelengths (Fig. 4). This is in agreement with the observations by Baird *et al.* [17] on the Hcs of chelicerates.

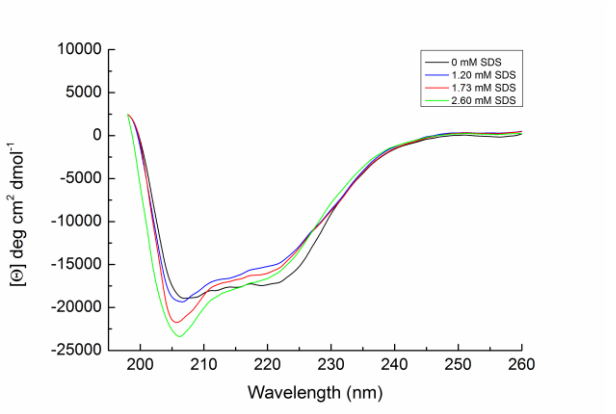


Fig. 4. Far-UV CD spectra of HaH in 25 mM phosphate buffer, pH 7.2, after 3 min incubation in different concentrations of SDS (1.2-2.6 mM).

Effect of SDS on the tertiary structure

SDS-induced changes in the tertiary structure of HaH were characterized by recording intrinsic fluorescence intensities and near-UV CD spectra. After excitation at 295 nm, the fluorescence spectrum of native HaH has maximum at 337±1 nm characteristic for tryptophans "buried" in the hydro phobic interior of the protein molecule. The fluorescence maxima of HaH, incubated in SDS (0.62-2.6 mM) for 3 min, were shifted to longer wavelength (Fig. 5). It appears that after treatment with SDS certain tryptophan side chains turn out to be exposed at the

Table 1. Kinetic parameters of intrinsic and induced *o*-diphenoloxidase activity of *Helix aspersa* maxima hemocyanin towards substrates catechol, L-Dopa and dopamine.

Hemocyanin	Catechol			L-Dopa			Dopamine		
	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (mM^{-1} min^{-1})	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (mM^{-1} min^{-1})	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (mM^{-1} min^{-1})
Native	11.8±0.5	1.1	0.093	no activity			no activity		
SDS-activated	0.91±0.23	31.45	34.56	15.26±0.98	9.58	0.62	0.51±0.17	31.08	60.94
Proteolytically activated	6.8±0.2	40.15	5.9	17.3±0.5	12.45	0.72			

surface of the protein molecule. The presence of SDS also led to increase of the intensity of fluorescence which is indicative for a reduction of internal quenching.

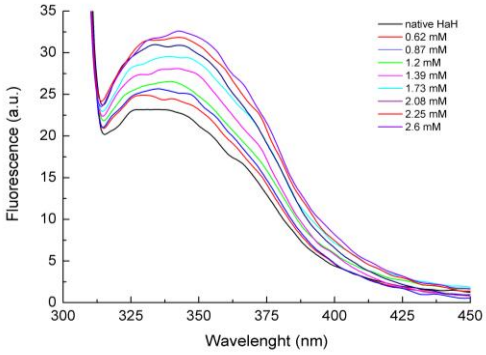


Fig. 5. Fluorescence spectra of HaH in 25 mM phosphate buffer, pH 7.2, excited at 295 nm, after 3 min incubation in different concentrations of SDS (0.62-2.6 mM).

Hcs and POs with bound dioxygen exhibit unique absorption spectra with a characteristic absorption peak at ~345 nm [5]. This absorption band results from the peroxide, which is present in a μ−η2:η2 side-on arrangement and acts as a strong σ-donor ligand to the Cu(II) ions. The di-copper center of Hcs is also responsible for the characteristic near UV-CD negative signal at 345 nm [18]. The intensity of the near-UV CD spectra (in the 260- to 320-nm range) of HaH, incubated in SDS (1.2-2.6 mM) for 3 min, was found to decrease substantially (Fig. 6), consistent with conformational changes that alter the microenvironment of at least some of the aromatic residues of the protein. Thus intrinsic fluorescence intensities and near-UV CD spectra both indicate changes in the tertiary structure of HaH upon treatment with SDS.

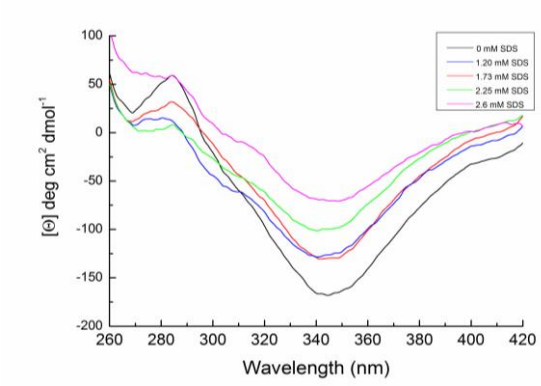
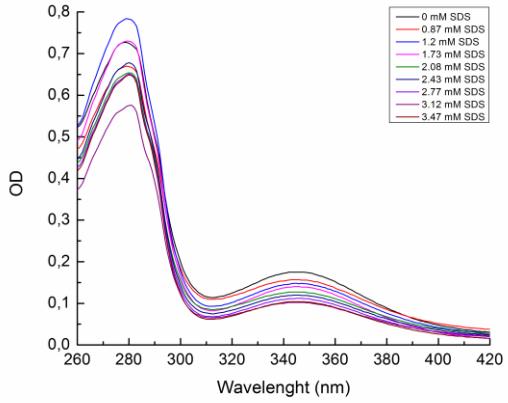
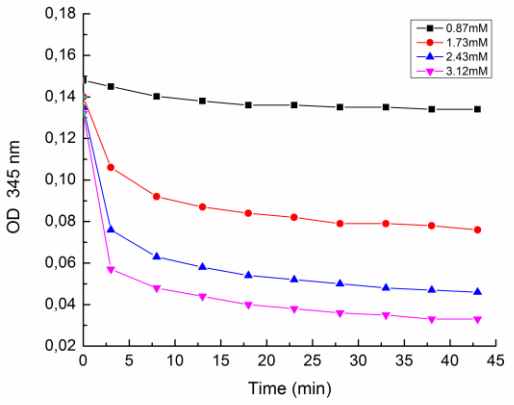


Fig. 6. Near-UV CD spectra of HaH in 25 mM phosphate buffer, pH 7.2, after 3 min incubation in different concentrations of SDS (1.2-2.6 mM).

Absorption spectra taken for HaH at increasing concentration (0.87-3.47 mM) of SDS showed that the intensity of copper-dioxygen band at 345 nm slightly decreased as a function of SDS concentration in the Hc samples (Fig. 7a, b). Therefore, SDS-induced conformational changes did not affect the integrity of the copper active sites in the Hc molecule.



(a)



(b)

Fig. 7. (a) Absorption spectra of HaH in 25 mM phosphate buffer, pH 7.2, after 3 min incubation in different concentrations of SDS (0.87-3.47 mM); (b) Absorbance at 345 nm of HaH in function of time in the presence of SDS at different concentrations (0.87-3.12 mM).

Assay of phenoloxidase activity

Kinetic analysis of the *o*-diPO activity of HaH, activated with SDS, with increasing concentrations of substrates catechol (from 0.1 to 1.2 mM), L-Dopa (from 0.5 to 12 mM) and dopamine (from 0.015 to 0.3 mM) was performed. The kinetic parameters (according to Michaelis-Menten kinetics), obtained by fitting the experimental data using the

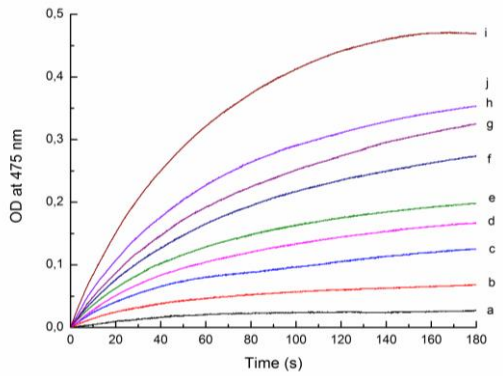
software HYPER, are presented in Table 1. High level of SDS-induced activity was detected for HaH towards substrate catechol – V_{max} increased up to 629 ± 0.002 nmol min^{-1} mg^{-1}, corresponding with a k_{cat} = 31.45 min^{-1} (Table 1). The enzymatic activity of HaH, treated with 1.73 mM SDS for 3 min, is related to that observed with proteolytically activated Hc (k_{cat} = 40.15 min^{-1}) [10]. However, the affinity of SDS-activated HaH towards substrate catechol is higher (K_m = 0.91 ± 0.2 mM) than the affinity shown by the native HaH (K_m = 11.8 ± 0.5 mM) and proteolytically activated HaH (K_m = 6.8 ± 0.2 mM) [10]. Moreover, the enzyme efficiency (k_{cat}/K_m = 34.56 mM^{-1} min^{-1}) of SDS-activated HaH towards catechol is considerably higher than that achieved for proteolytically activated Hc (k_{cat}/K_m = 5.9 mM^{-1} min^{-1}) [10]. This fact reveals that SDS-activated HaH possesses even higher potential for preparation of biosensors and studies are in progress. Recently, Naresh *et al.* report for a similar sensor for detection of phenols using Hc isolated from a freshwater gastropod *Pila globosa* [19]. The *o*-diPO activity of *P. globosa* Hc has also been enhanced by treatment with SDS.

After activation with SDS, HaH showed *o*-diPO towards the more bulky substrate L-Dopa and dopamine. The V_{max} value amounted to 192 ± 0.001 nmol min^{-1} mg^{-1}, k_{cat} = 9.58 min^{-1}, K_m value of 15.26 ± 0.98 mM characterizes the affinity of SDS activated HaH towards substrate L-Dopa. The highest *o*-diPO activity and enzyme efficiency of HaH (k_{cat}/K_m = 60.94 mM^{-1} min^{-1}), after incubation in 1.73 mM SDS for 3 min, was determined towards substrate dopamine (Fig. 8a, b). Dopamine was also found to be a preferred substrate of SDS-activated Hcs from arthropods *Cancer pagurus* [20] and *Eurypelma californicum* [21]. The physiological importance of this observation represents the fact that dopamine is important metabolite for sclera-tization and melanization of the cuticle.

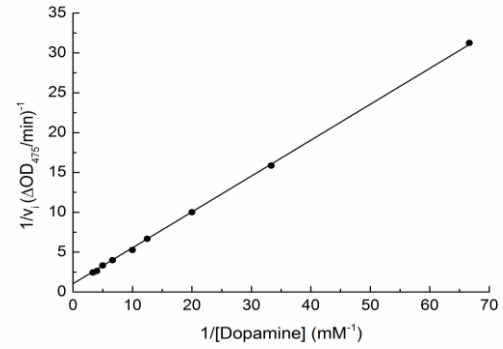
By means of electron cryomicroscopy Cong et al. reveal the structural mechanism of SDS-induced enzyme activity of Hc from the scorpion *Pandinus imperator* [22]. They show that secondary and tertiary structural alterations not only provide an increased access of substrates to active sites, but promote novel bridge formation between Hc linker subunits. In this way SDS seems to imitate an allosteric effector.

CONCLUSION

In conclusion, this study demonstrates that micellar concentration of detergent SDS can induced phenoloxidase activity in the hemocyanin of *Helix aspersa* maxima. The accompanying conformational changes in the protein result in opening a channel to the di-copper center allowing bulky phenolic substrates access to the catalytic site. The high enzyme efficiency of SDS-activated HaH towards catechol (k_{cat}/K_m = 34.56 mM^{-1} min^{-1}) and dopamine (k_{cat}/K_m = 60.94 mM^{-1} min^{-1}) reveals the benefit of utilization of PO activity of Hcs for preparation of Hc-based biosensors.



(a)



(b)

Fig. 8. (a) Kinetic analysis of *o*-diPO activity of HaH with increasing concentration of substrate dopamine: (a) 0.015 mM; (b) 0.03 mM; (c) 0.05 mM; (d) 0.08 mM; (e) 0.1 mM; (f) 0.15 mM; (g) 0.2 mM; (h).25 mM; (i) 0.3 mM. The assay was carried out in 25 mM phosphate buffer, pH 7.2, at 25°C and Hc concentration of 0.5 mg/ml; (b) The corresponding Lineweaver–Burk plot. The derived K_M and k_{cat} values are given in Table 1.

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SDS-ИНДУЦИРАНА ФЕНОЛОКСИДАЗНА АКТИВНОСТ НА ХЕМОЦИАНИН ОТ *HELIX ASPERSA* MAXIMA

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(Резюме)

Кислород-пренасящият протеин, изолиран от хемолимфата на охлюви от вида *Helix aspersa* maxima (HaH), беше превърнат във форма, която проявява фенолоксидазна активност. Посредством инкубиране на HaH с 1.73 mM SDS, в продължение на 3 мин, беше постигнато значително повишение на каталитичната ефективност на хемоцианина при субстрат катехол (k_{cat}/K_m = 34.56 $mM.min^{-1}$ срещу k_{cat}/K_m = 0.093 $mM.min^{-1}$, определена за нативен HaH). Най-висока SDS-индуцирана *o*-дифенолоксидазна активност и ефективност на HaH, след активиране посредством SDS, беше определена при субстрат допамин (k_{cat}/K_m = 60.94 mM^{-1} min^{-1}). Структурно характеризиране с помощта на абсорбционна и флуоресцентна спектроскопия и кръгов дихроизъм показаха, че мицеларен SDS предизвиква оптимални конформационни промени в протеина, в резултат на което активните центрове стават по-достъпни за молекулите на субстрата и хемоцианинът може да функционира като фенолоксидаза.