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Probing the two-domain structure of homodimeric prokaryotic and eukaryotic catalase–peroxidases

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Abstract

Catalase–peroxidases (KatGs) are ancestral bifunctional heme peroxidases found in archaeons, bacteria and lower eukaryotes. In contrast to homologous cytochrome *c* peroxidase (CcP) and ascorbate peroxidase (APx) homodimeric KatGs have a two-domain monomeric structure with a catalytic N-terminal heme domain and a C-terminal domain of high sequence and structural similarity but without obvious function. Nevertheless, without its C-terminal counterpart the N-terminal domain exhibits neither catalase nor peroxidase activity. Except some hybrid-type proteins all other members of the peroxidase–catalase superfamily lack this C-terminal domain. In order to probe the role of the two-domain monomeric structure for conformational and thermal stability urea and temperature-dependent unfolding experiments were performed by using UV–Vis-, electronic circular dichroism- and fluorescence spectroscopy, as well as differential scanning calorimetry. Recombinant prokaryotic (cyanobacterial KatG from *Synechocystis* sp. PCC6803) and eukaryotic (fungal KatG from *Magnaporthe grisea*) were investigated. The obtained data demonstrate that the conformational and thermal stability of bifunctional KatGs is significantly lower compared to homologous monofunctional peroxidases. The N- and C-terminal domains do not unfold independently. Differences between the cyanobacterial and the fungal enzyme are relatively small. Data will be discussed with respect to known structure and function of KatG, CcP and APx.

Keywords

Catalase–peroxidase; Two-domain structure; Gene duplication; Conformational stability; Thermal stability; Unfolding

1. Introduction

Catalase–peroxidases are heme-containing bifunctional oxidoreductases that probably evolved in ancestral Negibacteria [1] and later gave rise to divergent evolution of the very abundant peroxidase–catalase superfamily (formerly known as the “non-animal” heme peroxidase superfamily) [2,3]. The whole peroxidase–catalase superfamily is spread among both prokaryotic and eukaryotic cells but probably only catalase–peroxidase (KatG) retained its original twodomain monomeric structure and bifunctionality, i.e. catalase and peroxidase activity. In the course of ongoing evolution *katG* genes were shown to be transferred from

proteobacteria into genomes of Archae and most important also into genomes of lower eukaryotes by horizontal gene transfer (HGT) [4,5].

In principle, all 407 in PeroxiBase (<http://peroxibase.toulouse.inra.fr>, July 2010) annotated catalase–peroxidase sequences contain two well separated domains that arose via ancestral gene duplication [6,7]. Only the N-terminal domain which is in average 35% longer contains the prosthetic heme *b* group with no exception among known KatGs. In contrast, no heme binding motif was detected in any known C-terminal domain. Although all known C-terminal domains are shorter they reveal a high overall sequence similarity with their N-terminal counterparts [8]. The peculiar fold of the predominantly α -helices containing (N- and C-) domains has been preserved during evolution of the peroxidase–catalase superfamily. Currently four complete three-dimensional KatG structures are published [9–12] clearly demonstrating the above mentioned overall structure. In addition one structure of the C-terminal domain only is published [13]. The (N-terminal) heme cavity of KatG shows invariantly conserved amino acids found in all homologous members of the superfamily including cytochrome *c* peroxidase (CcP) and ascorbate peroxidase (APx) [10,14,15] and the active sites of these enzymes are virtually superimposable on one another with the exception of the KatG-specific distal adduct (Trp-Tyr-Met⁺) that is essential for the catalase activity of bifunctional KatG [15].

The role of the C-terminal domain in KatG is still under discussion. It does not catalyze any discernible reaction but seems to be essential for its bifunctional activity [16]. A KatG mutant containing only its N-terminal domain lacks catalase and peroxidase activities [16]. Results of site directed mutagenesis, kinetic and spectroscopic studies indicate that the distal (catalytic) histidine acts as heme ligand in the absence of the C-terminal domain, clearly indicating a structural cross-talk between the two KatG-specific domains. Finally, it has been demonstrated that separately expressed and isolated C-terminal domain is able to restructure the active site within the N-terminal domain thereby increasing the high-spin ferric state and recovering part of catalase and peroxidase activity [17].

In order to get more insight into the structural and functional role of the dimeric and two-domain monomeric structure of KatG [15], we have performed a comprehensive investigation of the conformational and thermal stability of KatGs from the cyanobacterium *Synechocystis* PCC6803 (*SynKatG*) and from the ascomycete *Magnaporthe grisea* (*MagKatG1*). The latter KatG was chosen since genome and phylogenetic analyses demonstrated the occurrence of *katG* genes also in lower eukaryotes, most prominently in sac and club fungi and because *MagKatG1* is the only eukaryotic representative that has been characterised in more detail so far [18]. Since inspection of the crystal structures of homologous Class I peroxidases (CcP, APx, KatG) [15] does not allow simple prediction of the role of the quaternary structure on the conformational and thermal stability of these metalloenzymes, unfolding of the corresponding recombinant prokaryotic and eukaryotic KatGs by either urea or temperature was followed by electronic UV–Vis-, circular dichroism (CD)-, and fluorescence spectroscopy as well as differential scanning calorimetry (DSC). Obtained data are discussed with respect to known 3D-structures of KatGs and homologous cytochrome *c* peroxidase and ascorbate peroxidases as well as with respect to differences in enzymatic activity.

2. Materials and methods

2.1. Expression and purification of recombinant KatGs

Recombinant catalase–peroxidases from *Magnaporthe grisea* and from *Synechocystis* PCC 6803 were expressed heterologously as described previously [18,19]. *Synechocystis* KatG was purified according to a standard protocol [19]. In the case of *Magnaporthe grisea*

cytosolic KatG (designated as KatG1 because this phytopathogenic fungus has also an extracellular KatG2) a modified purification procedure was applied. The first steps were performed as described in [18]. Active fractions collected from the Ni²⁺-loaded MCAC column were desalted on PD-10 columns (GE Healthcare) and applied onto a 25 ml hydroxyapatite column (Sigma Chemicals) pre-equilibrated with 5 mM sodium phosphate buffer, pH 7.4. *MagKatG1* was eluted stepwise by increasing the phosphate concentration up to 250 mM and selecting fractions with high-spin spectra and purity numbers ($A_{\text{Soret}}/A_{280 \text{ nm}} > 0.6$). Typical yield from 1 L of M9ZB was ~40 mg *SynKatG* and 30 mg *MagKatG1*.

2.2. Enzymatic activity

Catalase activity was determined spectrophotometrically by following H₂O₂ degradation at 240 nm ($\epsilon = 43.6 \text{ M}^{-1} \text{ cm}^{-1}$) [20]. One *catalatic* unit was defined as the amount of enzyme catalysing the conversion of 1 μmol of H₂O₂/min at an initial concentration of 15 mM H₂O₂ (pH 6.0, 25 °C). Peroxidase activity was monitored spectrophotometrically using 1 mM peroxyacetic acid and 5 mM guaiacol ($\epsilon_{470} = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$). One unit of peroxidase activity was defined as the amount that oxidises 1 μmol of guaiacol/min at pH 6 and 25 °C.

2.3. Monitoring of protein unfolding by urea

In unfolding studies with urea followed by UV–Vis spectroscopy (diode array spectrophotometer; Specord S10 from Carl Zeiss Jena; 10 mm path length quartz cuvettes), typically 2.2 μM of *Magnaporthe grisea KatG1* or 3.8 μM *Synechocystis KatG* in 5 mM phosphate buffer, pH 7.0, were incubated with various concentrations of urea (0–6M urea) for 18 h at 25 °C. Unfolding by urea in the near-UV and visible region (250–500 nm) was also monitored by ECD spectroscopy on PiStar-180 (Applied Photophysics, Leatherhead, U.K.) equipped with a thermostatic cell holder. The instrument was flushed with nitrogen at a flow rate of 5 L min⁻¹. Instrument parameters were set as follows: path length: 10 mm; spectral bandwidth: 5 nm; step size: 0.5 nm; scan time: 1315.9 s. Each spectrum was automatically corrected with the baseline to remove birefringence of the cell.

Furthermore, unfolding by urea was monitored by fluorescence spectroscopy using Hitachi F-4500 equipped with a thermostatic cell holder for quartz cuvettes of 10 mm path length. Changes in tryptophan emission were monitored using the instrumental parameters as follows. Excitation wavelength: 295 nm, excitation and emission bandwidth: 5 nm; PMT voltage: 700; scan speed: 60 nm min⁻¹. Typically, 500 nM of each protein in 5 mM phosphate buffer, pH 7.0, were incubated with increasing concentrations of urea (0–8M) for 18 h at 25°C. The content of tryptophans was calculated from protein sequences of *MagKatG1* (UniProt accession A4R5S9) and *SynKatG* (from the strain PCC 6803, UniProt accession Q79EX5).

2.4. Monitoring of protein unfolding by temperature

In thermal unfolding studies followed by UV–Vis spectroscopy 8.7 μM of *Magnaporthe grisea KatG1* or 7.95 μM *Synechocystis KatG* in 5 mM phosphate buffer, pH 7.0, containing 0.5 M urea were incubated at increasing temperature ranging from 20 to 60 °C. Non-denaturing concentration of urea (0.5 M) was added to the buffer in order to avoid unspecific aggregation of protein at higher temperature. Experiments were performed using Chirascan (Applied Photophysics, Leatherhead, U.K.) that allowed simultaneous UV–Vis and CD monitoring and was equipped with a peltier temperature control unit for stepwise temperature increase (2 °C) at defined incubation times (30 s–2 min). For monitoring ECD spectra as a function of temperature Chirascan was flushed with nitrogen with a flow rate of 5 L min⁻¹. In the near-UV and visible region (250–500 nm) instrument parameters were set as follows: path length: 10 mm; spectral bandwidth: 1 nm; step size: 1 nm; scan time per

point: 0.5 s (scan period: 25 μ s $\times$ 20,000 counts); scan time: $\sim$ 125 s. In addition single wavelength scans at 412 nm (Soret region) were performed with the same instrumental set up mentioned above. Temperature-mediated changes in the far-UV region were recorded by following changes in ellipticity at 222 nm. Path length: 1 mm, spectral bandwidth: 0.5 nm, scan time per point: 12 s (scan period: 25 μ s $\times$ 480,000 counts).

2.5. Differential scanning calorimetry

DSC measurements were performed using a VP-DSC microcalorimeter from Microcal, controlled by the VP-viewer programme and equipped with a 0.51 ml cell. Studies were made with 4 μ M *MagKatGI* or *SynkatG* in 5 mM phosphate buffer, pH 7, containing 0.5 M urea in order to avoid unspecific aggregation at higher temperature. Samples were analyzed using a programmed heating scan rate of 90 $^{\circ}$ C h $^{-1}$ (1.5 $^{\circ}$ C min $^{-1}$) over a temperature range of 20 to 60 $^{\circ}$ C and approximately 30 psi (2.068 bar) cell pressure. Collected DSC data were corrected for buffer baseline and normalized for protein concentration. The heat capacity (C_p) was expressed in kcal mol $^{-1}$ K $^{-1}$ (1 cal=4.184 J). For data analysis and conversion the Microcal origin software was used. Data points were fitted to both two-state and non-two-state equilibrium-unfolding models by the Lavenberg/Marquardt (LM) non-linear least square method.

2.6. Calculation of thermodynamic parameters

The fraction α of unfolded protein at defined urea concentration or temperature was calculated from the absorbance at the Soret maximum of the native enzyme (408 nm) according to $\alpha=(A_N-A)/(A_N-A_U)$, with A representing the absorbance at defined urea concentration or temperature (T), A_N the absorbance at 408 nm of the native state and A_U the absorbance at 408 nm of the unfolded state. Similarly, from ECD data the fraction α of unfolded protein α was calculated according to $\alpha=(\theta_N-\theta)/(\theta_N-\theta_U)$ with θ_N being the ellipticity at 222 nm (α -helix minimum) or 412 nm (Soret minimum) of the protein in the native folded state, θ the ellipticity at defined urea concentration or temperature (T), and θ_U being the ellipticity at 222 nm or 412 nm of the completely unfolded state. In fluorescence studies $\alpha=(F_N-F)/(F_N-F_U)$, with F_N representing the fluorescence emission maximum of the native state, F being the emission maximum at defined urea concentration and F_U the emission maximum of the completely unfolded state.

Assuming a two-state transition $N \rightleftharpoons U$ [21], where N is the native protein and U is the unfolded protein, the equilibrium constant between the unfolded and native state is given by $K=[U]/[N]=\alpha/(1-\alpha)$ and is related to the standard free energy of denaturation according to $\Delta G^{\circ}=-RT\ln K$, where R is the universal gas constant (8.31 JK $^{-1}$ mol $^{-1}$) and T is the absolute temperature. In unfolding experiments with chaotropic reagents performed at 25 $^{\circ}$ C (298.13 K) ΔG° and K depend on the denaturant concentration according to the linear extrapolation method [22]: $\Delta G^{\circ}=\Delta G^{\circ}_{H_2O}-m[\text{denaturant}]$, where $\Delta G^{\circ}_{H_2O}$ is the value of ΔG° at zero concentration of denaturant (i.e. the conformational stability of a protein) and m reflects the efficacy of the denaturant in unfolding and largely depends on the size and composition of the polypeptide chain exposed to the solvent.

In thermal unfolding experiments, changes in enthalpy, ΔH_T , and entropy, ΔS_T , at a given temperature T , are related with the change in standard free energy according to $\Delta G^{\circ}_T = -RT\ln K_T = \Delta H_T - T\Delta S_T$. Upon assuming that change in heat capacity at constant pressure (ΔC_p) is independent of temperature, it follows that $\Delta H_T = \Delta H_m + \Delta C_p(T - T_m)$, and $\Delta S_T = \Delta S_m + \Delta C_p \ln(T/T_m)$, respectively, with ΔH_m and ΔS_m representing changes in enthalpy and entropy at midpoint transition, i.e. when $T = T_m$. This allows calculation of ΔG°_T at various temperatures [22].

$\Delta G^\circ_T = \Delta H_T - T\Delta S_T = \Delta H_m + \Delta C_p (T - T_m) - T[\Delta S_m + \Delta C_p \ln (T/T_m)]$. Upon using $\Delta S_m = \Delta H_m/T_m$ it follows that $\Delta G^\circ_T = \Delta H_m + \Delta C_p (T - T_m) - T[\Delta H_m/T_m + \Delta C_p \ln (T/T_m)]$ and, finally, $\Delta G_T = \Delta H_m (1 - T/T_m) + \Delta C_p [T - T_m - T \ln(T/T_m)]$. At midpoint transition, $T = T_m$, and $\Delta G^\circ_T = \Delta G^\circ_m = 0$.

From $\Delta G^\circ_T = -RT \ln K_T = \Delta H_m - T\Delta S_m$ it follows $-R \ln K_T = \Delta H_m/T - \Delta S_m$ and $R \ln K_T = \Delta S_m - \Delta H_m/T$ allowing calculation of ΔH_m and ΔS_m from a linear plot of $R \ln K_T$ versus $1/T$ (van't Hoff plot). The value of total heat capacity change, ΔC_p , was obtained

$$\text{from } \Delta C_p = \frac{\Delta H_T - \Delta H_m}{(T - T_m)}.$$

3. Results and discussion

Electronic UV–Vis spectra of both recombinant *Magnaporthe grisea* KatG1 and *Synechocystis* KatG at pH 7 exhibit spectral properties typical for a mainly five-coordinated high-spin heme iron [14]. The Soret band of *MagKatG1* was observed at 408 nm with additional specific Q-bands at 504 and 546 nm, and a CT1 band at 635 nm. This compares with the corresponding *SynKatG* absorption maxima at 408 nm, 502, 542 and 637 nm.

3.1. Urea-mediated unfolding

Fig. 1 compares the effect of urea at 25 °C on the integrity of the heme cavity of *MagKatG1* and *SynKatG* as determined by UV–Vis spectroscopy in the Soret region. It clearly depicts that heme *b* of both (prokaryotic and eukaryotic) KatGs is easily released from the active site at urea concentrations between 2.0 and 2.5 M. In the beginning (< 2 M urea) the Soret band maximum became slightly (~2 nm) redshifted and sharpened (accompanied by loss in intensity) suggesting that urea can enter the main access channel and approach the heme iron thereby acting as a high-spin ligand. This phenomenon has also been observed with other heme peroxidases and precedes the actual unfolding process [23]. Below 2 M urea both the catalase and peroxidase activity slightly decreased in both proteins to the same extent [e.g. *MagKatG1* catalase activity: 100% (0 M urea), 96% (1 M), 82% (2 M); *MagKatG1* peroxidase activity: 100% (0 M urea), 97% (1 M), 86% (2 M)]. Between 2.0 M and 2.5 M urea the Soret maximum in both proteins shifted abruptly to 369 nm accompanied by peak broadening, further loss in intensity and complete loss of bifunctional activity (Fig. 1C and D). This is caused by release of (free) heme from the protein due to significant urea-mediated structural changes at the active site. There seems to be no intermediate state as is obvious from the presence of an isosbestic point in transition of Soret spectra of both proteins (Fig. 1A and B) as well as from the plots depicted in Fig. 1C and D. This differs KatG from homologous cytochrome *c* peroxidase (CcP, together with KatG a Class I peroxidase) where an intermediate was observed during urea-mediated unfolding [23]. Moreover, in (monofunctional) CcP, that has lost the C-terminal (KatG-typical) domain during evolution, formation of free heme is observed at urea concentrations >4 M. In horseradish peroxidase, a secretory Class III peroxidase, the heme cavity seems to be even more stable toward urea than in CcP [23].

Data presented in Fig. 1C and D suggest a two-state transition for both KatGs. From the plots of $A_{408 \text{ nm}}$ versus urea concentration C_m -values (midpoint transition with $K = [U] / [N] = 1$) could be calculated to be 2.0 M (*MagKatG1*) and 1.5 M (*SynKatG*). From linear plots of standard free enthalpies versus urea concentration (Fig. 1E and F) the conformational stability, $\Delta G^\circ_{H_2O}$, of the heme cavity of *MagKatG1* (6.4 kJ mol⁻¹) and *SynKatG* (9.6 kJ mol⁻¹) could be calculated (25 °C, pH 7). The lower C_m determined for *SynKatG* can be explained by the higher *m*-value (Table 1) that might reflect differences in size and composition of the polypeptide chains exposed to the solvent and thus in efficacy of urea to mediate unfolding of the two proteins.

In addition heme ECD in relation to various urea concentrations was analysed. Free heme does not show circular dichroism whereas in an asymmetric (protein) environment it exhibits pronounced either positive or negative Soret peaks [24,25]. In native *MagKatG1* (Fig. 2A) and *SynKatG* (not shown) ECD spectra show a prominent minimum at 412 nm that loses ellipticity with increasing urea concentration. Similarly, the (positive) band around 376 nm and the broad (negative) band in the near-UV region lose ellipticity. Unfolding follows a simple two-state transition (Fig. 2C) leading to complete loss of ellipticity. Heme-CD monitored denaturation is a more sensitive probe than heme UV–Vis spectroscopy. Loss of ellipticity occurs already at low urea concentration (Fig. 2A and C). Nevertheless, calculated $\Delta G^{\circ}_{H_2O}$ (as well as C_m and m) values were very similar to those obtained from monitoring the heme absorbance (Table 1).

Finally, urea-mediated denaturation of the overall KatG structures was investigated by measuring the steady-state intrinsic fluorescence of tryptophans at defined urea concentrations. In heme proteins quenching of Trp fluorescence can be caused by the prosthetic group [26]. In KatG this concerns the N-terminal domain that has also a higher Trp content compared to the C-terminal domain [*MagKatG1*: N-terminal domain (423 aa, 15 Trp), C-terminal domain (327 aa, 7 Trp); *SynKatG*: N-terminal domain (436 aa, 19 Trp), C-terminal domain (318 aa, 7 Trp)]. Relief of heme quenching, as well as a redshift in the steady-state tryptophan fluorescence is observed when both KatGs were incubated with increasing urea concentrations at pH 7.0. These observations are indicative of protein unfolding and Trp exposure to the aqueous environment (Fig. 3) [27]. The emission maximum gradually shifts from 335 ± 2 nm in the absence of urea to 351 ± 2 nm at highest urea concentration (8 M urea). It is interesting to note that in native CcP the emission maximum is blue-shifted (325 nm) [23] compared to both native KatGs (~ 335 nm) suggesting a more compact structure in CcP compared to KatG.

Upon plotting the fluorescence emission maximum *versus* urea concentration it can be seen that both KatGs do not alter their overall structure by a simple two-state process (Fig. 3C and D). The biphasic transition is more pronounced in the prokaryotic protein (Fig. 3D) allowing the calculation of two separated sets of thermodynamic data (Table 1). Inspection of the plot in Fig. 3D suggests the existence of an intermediate state between 1.5 and 2.0 M urea in *SynKatG*. Since major changes in Soret absorbance and heme-ECD occurred at [urea] < 2.5 M, it is reasonable to assume that the first phase corresponds mainly to unfolding of the N-terminal domain of *SynKatG* (Fig. 3B, gray spectra). The corresponding conformational stability was calculated to be 3.1 kJ mol^{-1} . The second phase of transition occurred at [urea] > 2.5 M (where the heme prosthetic group is known to be released completely, see above) and can be assigned mainly to conformational changes in the C-terminal domain with $\Delta G^{\circ}_{H_2O} = 6.3 \text{ kJ mol}^{-1}$ (Fig. 3D). The less pronounced transition in *MagKatG1* did not allow separation of the two phases nor calculation of values from the data set. Values in Table 1 refer to the second transition (i.e. the C-terminal domain). In any case the relatively low conformational stability of the N-terminal domain of KatG indicates weak hydrophobic packing despite the high Trp content. Interestingly typical heme catalase from yeast showed a comparable low conformational stability [28].

3.2. Temperature-mediated unfolding

Furthermore, the thermostability of the active site was investigated by Soret UV–Vis and CD spectroscopy. Fig. 4A and B demonstrate a direct (monophasic) temperature-mediated blue-shift of the Soret maximum from 408 nm to 370 ± 1 nm with isosbestic points at 383 nm (*MagKatG1*) and 387 nm (*SynKatG*). The shift in the Soret band was associated by a decrease in the Soret band intensity and loss of specific bands (Q and CT1 bands) (not shown). Similar to urea-mediated unfolding the change in Soret maximum was abrupt and occurred within a few degrees (Fig. 4C and D). Loss in Soret intensity precedes change in

Soret wavelength maximum and from the corresponding plots the midpoint of transition (T_m) was calculated to be 41.3 °C (*MagKatG1*) and 43.0 °C (*SynKatG*), respectively. Very similar results were obtained by following the (monophasic) loss of ellipticity at 412 nm as a function of temperature ($T_m = 41.4^\circ$ and 43.5 °C, respectively) (Fig. 2B and D).

The thermodynamic parameters $\Delta G^\circ_{H_2O}$, as well as ΔH_m , ΔS_m , $T_m (= \Delta H_m / \Delta S_m)$ and ΔC_p for thermal unfolding were obtained from the fits to the stability curve (ΔG° versus temperature) and to the corresponding van't Hoff plots (inset to Figs. 2D and 4E and F). Very similar data were obtained for both proteins, suggesting that the temperature sensitivity of the heme cavity is similar in prokaryotic and eukaryotic KatGs.

Fig. 5A and B represent the change in far-UV spectra (190–250 nm) upon increase in temperature. In both native *MagKatG1* and *SynKatG* the dominating α -helical fold is evident by two minima at 208 and 222 nm. With increasing temperature both minima loose ellipticity and merge to a single minimum around 215 nm. Both KatGs show characteristic residual CD intensity when unfolded by heat as was seen with many other proteins including peroxidases [29–31]. From the plots of $[\theta]_{222}$ versus temperature (Fig. 5C and D) T_m values of 43.7 (*MagKatG1*) and 44.0 °C (*SynKatG*) were calculated, slightly higher than those estimated for heme cavity unfolding (Table 2). This indicates that temperature-mediated disruption of the heme cavity and heme release as well as unfolding of the α -helices occur in a concerted way. The overall thermal stability of *MagKatG1* and *SynKatG* was very similar and in both proteins temperature-mediated unfolding was irreversible. In contrast to urea-mediated unfolding followed by fluorescence spectroscopy, the temperature susceptibility of far-UV ECD did not exclude a two-state transition.

Additionally, calorimetric studies of the thermal denaturation of both KatGs were performed. DSC profiles for *MagKatG1* and *SynKatG* in 5 mM phosphate buffer, pH 7.0, containing 0.5 M urea are shown in Fig. 6. With both proteins one single endotherm was obtained with maxima at 45 °C (*MagKatG1*) and 48 °C (*SynKatG*). In the absence of urea both proteins were prone to aggregation at $T > 45^\circ\text{C}$, but in the presence of 0.5 M urea no exothermic transition typical for protein aggregation could be observed. The unfolding transitions were irreversible and did not show any detectable thermogram upon rescanning. DSC data could not be fitted very well on the basis of a single two-state transition model. Deconvolution of data might propose a non-two-state unfolding model and the presence of two distinct and separate domains with two close maxima for *MagKatG1* (43.0 °C and 45.8 °C) and *SynKatG* (44.5 °C and 48.1 °C).

In contrast to KatG homologous cytochrome *c* peroxidase shows two well separated endotherms observed by DSC during thermal denaturation at pH 7.0, with the first transition temperature (43.9 °C) being similar to that observed for KatG and the second being at 63.3 °C [32]. Only one single endotherm was observed for the unfolding of the apoprotein, and this transition was similar to the high-temperature transition in the holoenzyme. This reflects the urea-mediated unfolding data of CcP that also demonstrated a clear two-phasic unfolding behaviour of CcP [23]. Data were explained by the fact that (monomeric) CcP is folded in two domains with the heme bound in a cleft between the two domains. The N-terminal domain I is more loosely organized in CcP with about half of the main-chain atoms having high values of the isotropic thermal parameter, indicating either greater statistic disorder or larger thermal motion [32]. It was suggested that part of domain I is unfolded in the low-temperature transition thereby causing shift of the Soret band from 408 to 414 nm, which was attributed to binding of a distal residue to the heme. Loss of secondary structure did not accompany this transition. In the second phase more rigid parts of domain I as well as C-terminal domain II unfold and heme is released completely [32]. By contrast, temperature-mediated unfolding of (homodimeric) ascorbate peroxidase followed by DSC showed only

one endotherm with a T_m of 58.3 °C [33]. Release of heme followed the same cooperative two-state transition as melting of secondary structures ($T_m = 57.9$ °C).

The presented findings demonstrate significant differences in conformational and thermal stability of homologous Class I peroxidases, although principally KatG, CcP and APx show a high degree of similarity of secondary and tertiary structural elements including the architecture of the respective heme cavities. However, KatGs exhibit the lowest percentage in α -helices and the highest in short helices and loops [15]. The latter are typical for KatG and connect the distal and proximal heme sides of the N-terminal domain as well as contribute to the architecture of the long and restricted access channel that is important for the bifunctional activity of KatG. The presence of these large (flexible) loops might be responsible that catalase–peroxidases are significantly more prone to unfolding compared to both CcP and APx. Relatively low concentrations of urea as well as low temperatures caused abrupt and total heme release and inactivation of this unique bifunctional peroxidase.

Furthermore far-UV and DSC data suggest that the KatG-typical N- and C-terminal domains do not unfold independently and have very similar thermal stabilities. A slightly lower stability of the N-terminal domain can be proposed from overall unfolding studies by urea in addition to UV–Vis and ECD studies in the Soret region. In any case, the obtained data well reflect investigations about the role of the C-terminal domain in the activity of KatG [16,17]. They suggest that the C-terminal domain is an integral component of the KatG structure and function. In its absence the active site is in a hexacoordinate low-spin state [17]. Visual inspection of the KatG structures [10–13] show prominent intra- and intersubunit interactions between distinct loops and helices of the N- and C-terminal domains and between the monomers. These points of contacts between the two domains and monomers contribute to the stability of the overall quaternary structure. It has also been suggested that the C-terminal domain might serve as a platform to ensure proper folding of the N-terminal domain [34].

It is interesting to note that the catalase activity of KatG is very sensible to structural changes at the active site or in the substrate channel. Most of the so far designed mutants showed reduced catalase activity, whereas the peroxidase activity was unaffected or even enhanced by the manipulations [14,15,35]. This seems to be contradictory to the fact that KatG is a very labile protein with low conformational and thermal stability. Apparently, the *catalatic* activity of KatG functions within a small range of flexibility and structural integrity. From an evolutionary point of view KatG is a predecessor of APx and CcP [15], but the exact evolutionary lineage of eukaryotic CcP and APx is still under discussion. It is interesting to note that in a recently detected hybrid-type peroxidase [15] a second (C-terminal) domain was also found [36]. In any way, the C-terminal domains of prokaryotic KatGs were lost in the majority of (eukaryotic) genes of CcP and APx with simultaneous loss of the KatG-typical loops. As a consequence their conformational and thermal stability has increased. Both CcP and APx are typical monofunctional peroxidases that have completely lost the capacity to efficiently dismutate hydrogen peroxide.

This paper also demonstrates that fungal and bacterial catalase–peroxidases have a comparable conformational and thermal stability. This is underlined by the fact that during the evolutionary history KatGs were acquired by ancestral fungi from bacterial genomes via horizontal gene transfer in an already developed state. The fact that the overall structure and conformational and thermal stability has been strictly conserved in eukaryotic KatGs additionally supports its importance in bifunctional activity.

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Abbreviations

KatG	catalase–peroxidase
MagKatG1	cytosolic KatG from <i>Magna-porthe grisea</i>
SynKatG	KatG from <i>Synechocystis</i> PCC6803
CcP	cytochrome <i>c</i> peroxidase
APx	ascorbate peroxidase
HRP	horseradish peroxidase
HGT	horizontal gene transfer
ECD	electronic circular dichroism
DSC	differential scanning calorimetry
aa	amino acid

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Fig. 1. Monitoring urea-mediated denaturation of *Magnaporthe grisea* KatG1 (A, C, E) and *Synechocystis* KatG (B, D, F) by heme Soret spectroscopy. (A) Spectral transition of 2.2 μM recombinant *Magnaporthe grisea* KatG1 in 5 mM phosphate buffer, pH 7.0, mediated by various urea concentrations (incubation time 18 h at 25 $^\circ\text{C}$): 0 M (gray line), 1.5, 2, 2.5 and 5 M). (B) Spectral transition of 3.8 μM recombinant *Synechocystis* KatG in 5 mM phosphate buffer, pH 7.0, mediated by various urea concentrations (incubation time 18 h at 25 $^\circ\text{C}$): 0 M (gray line), 1.0, 1.5, 2.0 and 3.5 M urea). (C, D) Plots of Soret maximum and absorbance at 408 nm in dependence of urea concentration. Conditions as in (A, B). (E, F) Standard free enthalpies as a function of urea concentration. ΔG° values have been calculated from plots of absorbance at 408 nm versus urea concentration as described in Materials and methods.

Fig. 2.

Monitoring urea- and temperature-mediated unfolding of KatG from *Magnaporthe grisea* by heme electronic circular dichroism (ECD) spectroscopy. (A) Soret circular dichroism spectra of 6 μM native *Magnaporthe grisea* KatG (gray line) incubated with various concentration of urea (0.5, 1, 1.25, 1.5, 1.75, 2, 2.25 and 6 M) for 18 h at 25 $^{\circ}\text{C}$. Panel (B) shows the changes in ECD spectra during the thermal transition of *Magnaporthe grisea* KatG (8.7 μM) in 5 mM phosphate buffer, pH 7.0, containing 0.5 M urea. The ECD spectrum at 25 $^{\circ}\text{C}$ is depicted in gray. Panel (C) shows the change in CD ellipticity at 412 nm as a function of denaturant concentration for *MagKatG*. Inset shows the change in standard free enthalpy with urea concentration. Panel (D) along with the insets shows the loss of CD ellipticity at 412 nm in *MagKatG1* with increasing temperature and the corresponding Van't Hoff plot.

Fig. 3.

Denaturation of *Magnaporthe grisea* KatG1 and *Synechocystis* KatG by urea as monitored by fluorescence spectroscopy. (A) Fluorescence emission spectra of *Magnaporthe grisea* KatG (0.5 μ M) in 5 mM phosphate buffer, pH 7.0, upon 18 h (25 $^\circ$ C) incubation with various concentrations of urea [0 M (gray line), 2.25, 2.5, 3.5, 4, 5.25, 7.25 and 7.8 M]. (B) Fluorescence emission spectra of *Synechocystis* KatG (0.5 μ M) in 5 mM phosphate buffer, pH 7.0, upon 18 h (25 $^\circ$ C) incubation with various concentrations of urea [0 M (gray line), 0.25, 0.5, 1, 1.75, 3, 5, 6.5, and 7.5 M]. (C, D) Plot of change in Trp emission maximum *versus* urea concentration. Conditions as in (A, B). The inset depicts the corresponding stability curve (ΔG° versus urea concentration) and fit for the second transition. Gray spectra and circles indicate first transition (2.25 M urea), black spectra and circles represent second transition. Excitation: 295 nm, emission range: 300–450 nm.

Fig. 4. Thermal stability of the heme cavity of *Magnaporthe grisea* KatG1 and *Synechocystis* KatG as monitored by electronic absorbance spectroscopy. Change in heme Soret spectra during thermal denaturation of *MagKatG* (A) and *SynKatG* (B). (C, D) Change in Soret maximum (●) and decrease of Soret intensity (○, at 408 nm) of *Magnaporthe grisea* KatG (8.7 μM) and *Synechocystis* KatG (7.95 μM) in 5 mM phosphate buffer, pH 7.0, containing 0.5 M urea. (E, F) Van't Hoff plots and fits.

Fig. 5.

Overall thermal stability of *Magnaporthe grisea* KatG1 and *Synechocystis* KatG followed by far-UV ECD. Changes in ECD spectra of 5 μ M *Magnaporthe grisea* KatG1 (A) and 5 μ M *Synechocystis* KatG (B) with increasing temperature. Buffer: 5 mM phosphate buffer, pH 7.0, containing 0.5 M urea. (C, D) Corresponding changes in ellipticity at 222 nm in *MagKatG1* (C) and *SynKatG* (D). Insets: Van't Hoff plots and fits.

Fig. 6.

Differential scanning calorimetry of *Magnaporthe grisea* KatG1 and *Synechocystis* KatG and dimeric structure of KatG. Normalised DSC thermograms of *MagKatG1* (A) and *SynKatG* (B) after pre and post-translational baseline subtraction. Conditions: 4 μ M enzyme in 5 mM phosphate buffer, pH 7.0, containing 0.5 M urea. Fit of experimental data points (gray line) to a non-two-state model with two unfolding transitions shown in black. (C) Dimeric structure of *Burkholderia pseudomallei* KatG (PDB code: 1MWV). Blue and orange: heme-containing N-terminal domains, red and green: C-terminal domains. Figure was constructed with PyMOL.

Table 1

Thermodynamic parameters derived from urea-mediated unfolding studies of catalase-peroxidase from *Magnaporthe grisea* (*MagKatG1*) and *Synechocystis* PCC6803 (*SynKatG*).

Methods	$\Delta G^{\circ}_{H_2O}$ (kJ mol ⁻¹)		m (kJ mol ⁻¹ M ⁻¹)		C_m (M)	
	MagKatG1	SynKatG	MagKatG1	SynKatG	MagKatG1	SynKatG
UV-vis	6.4	9.6	3.2	6.5	2.0	1.5
[θ] ₄₁₂	6.2	10.5	4.9	7.2	1.3	1.5
Trp fluorescence. <i>N-terminal domain</i>	n.c.	3.1	n.c.	6.8	n.c.	0.5
Trp fluorescence. <i>C-terminal domain</i>	6.5	6.3	2.3	2.6	2.8	2.4

$\Delta G^{\circ}_{H_2O}$, conformational stability³⁴; m , efficacy of urea in unfolding; C_m , urea concentration with $K=[U]/[N]=1$, n.c., could not be calculated from experimental data.

Table 2

Thermodynamic parameters derived from thermal unfolding studies of catalase-peroxidase from *Magnaporthe grisea* (MagKatG1) and *Synechocystis* PCC6803 followed by UV-Vis-(Soret region), circular dichroism (CD, Soret and far-UV region) and differential scanning calorimetry (DSC).

Method	$\Delta G^{\circ}_{H_2O}$ (kJ mol ⁻¹)		ΔH_m (kJ mol ⁻¹)		ΔS_m (kJ mol ⁻¹ K ⁻¹)		C_p (kJ mol ⁻¹ K ⁻¹)		T_m (°C)	
	MagKatG1	SynKatG	MagKatG1	SynKatG	MagKatG1	SynKatG	MagKatG1	SynKatG	MagKatG1	SynKatG
UV-Vis	8.3	8.5	231.5	234.6	0.73	0.74	9.2	9.1	41.3	43.0
[θ] ₄₁₂	8.9	8.6	236.2	234.7	0.75	0.74	9.5	9.2	41.4	43.5
[θ] ₂₂₂	8.2	8.5	232.9	234.0	0.73	0.73	9.3	9.4	43.7	44.0
DSC	47.2	56.9	850.9	978.6	2.66	3.04	8.4	8.0	45.0	48.1
N-terminal domain	19.5	25.0	372.6	456.7	1.17	1.43	–	–	43.0	44.5
C-terminal domain	27.6	31.8	478.3	521.9	1.49	1.62	–	–	45.8	48.1

$\Delta G^{\circ}_{H_2O}$, conformational stability; ΔH_m , ΔS_m , enthalpic and entropic changes at midpoint transition ($T = T_m$).