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Increase of the peroxidase activity of cytochrome c-550 by the interaction with detergents

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Abstract

The effect was studied of detergents on the peroxidase activity of the heme-containing electron-transport protein, cytochrome c-550 from *Paracoccus versutus* (cytc550). Cytc550 does not interact with non-ionic or zwitterionic detergents, but its peroxidase activity is significantly enhanced in the presence of both anionic and cationic detergents. The increase in peroxidase activity is caused by (partial) unfolding of cytc550, resulting in weakening of the bond between the axial methionine ligand and the heme-iron. With sodium dodecyl sulfate (SDS) and dodecyltrimethylammonium bromide (DTAB), the activity increases roughly 10- and 100-fold, respectively. The detergent concentration required to enhance the peroxidase activity of cytc550 consistently coincides with the critical micellisation concentration (CMC). When cytc550 carries substantial opposite charge to that of the detergent headgroup, the protein–detergent complex is formed at lower detergent concentration than the critical micellisation concentration, i.e. at acid pH for anionic detergent and at alkaline pH for cationic detergent. It is concluded that in the presence of ionic detergents, cytc550 can acquire considerable peroxidase activity. This may be exploited by applying cytc550 as an oxidative catalyst in detergent-rich environments, where regular peroxidases rapidly lose their catalytic potential.

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Keywords: Cytochrome c; Peroxidase activity; Critical micellisation concentration (CMC); Surfactants

1. Introduction

In view of the contemporary need for more environmentally friendly production processes in chemistry, so-called ‘green chemistry’, there is considerable interest in the application of enzymes as catalysts [1]. Of these, peroxidases are interesting candidates because they function in a variety of oxidation reactions on a broad spectrum of substrates [2,3]. Peroxidases are heme-containing enzymes that efficiently catalyse substrate oxidations using the environmentally ‘clean’ oxidant hydrogen peroxide.

Cytochrome c-550 from *Paracoccus versutus* (cytc550) is a heme-protein that in vivo functions as an electron transporter, although in vitro it also possesses a low activity as a peroxidase [4–6]. In cytc550, the heme-cofactor, which is essential for catalysis, is covalently bound to the protein through thioether bonds to two cysteine residues. This gives rise to the interesting phenomenon that when the struc-

ture of cytc550 is perturbed, e.g. by unfolding, the peroxidase activity increases considerably and unfolding renders cytc550 a competent peroxidase [7,8]. Cytc550 can thus, in principle, function as a peroxidase under conditions where the protein is unfolded, i.e. under conditions normally considered harsh for proteins. Under such conditions, the use of natural peroxidase enzymes is limited, because these enzymes lose their heme-cofactor when the protein unfolds [9–11].

Previously, we reported on the effect of a chaotropic agent (guanidinium hydrochloride) on enhancing the peroxidase activity of cytc550 [7,8]. Here we study the effect of detergents on the cytc550 peroxidase activity. In industry, the use of detergents is as ubiquitous as it is important [12], and for this reason it is relevant to understand in more detail the possible application of cytc550 as a peroxidase in the presence of detergents.

It is demonstrated that non-ionic or zwitterionic detergents have no effect on the peroxidase activity, while the anionic detergent sodium dodecyl sulfate (SDS) and cationic detergents containing quaternary ammonium headgroups enhance the peroxidase activity of cytc550. Efficient binding by both

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types of ionic detergents is related to the high surface charge and dipolar nature of cytc550. The peroxidase activity increase is consistently observed to occur close to the critical micellisation concentration (CMC) of the detergent. The increase in peroxidase activity brought about by SDS is moderate, and cationic detergents cause a significantly higher increase in the peroxidase activity of cytc550 (up to ~100 times). This indicates that in the presence of detergents, cytc550 may be suitable as a 'green' oxidation catalyst.

2. Experimental section

P. versutus cytc550 was produced and isolated as before [6]. The ferric form of cytc550 was used throughout. The detergents used were all highest grade from Sigma, except for dodecyltrimethylammonium bromide (DTAB, Fluka, Chemika grade) and SDS (Fluka, Biochemika grade). 1-*N*-phenyl-naphthylamine (NPN) was from Sigma, as was the peroxidase substrate guaiacol (*o*-methoxyphenol). All experiments were performed in the presence of 100 mM sodium phosphate, except when indicated otherwise. All experiments were performed at 293 K and measurements were performed after equilibrating the cytc550 with detergent >3 h, to ensure equilibrium conditions.

The peroxidase activity of cytc550 as a function of detergent concentration was measured in a plastic 1.5 ml cuvette, by mixing 680 μ l of cytc550-containing solution with 120 μ l of solution containing both guaiacol and H₂O₂ (with identical [detergent] in each solution). Given the limited solubility of guaiacol in water, this was the optimal ratio to minimise the difference in free detergent concentration in the protein-containing solution and the reaction mixture. In the final reaction mixture, [cytc550] was 0.61 or 4.4 μ M, [guaiacol] was 10 mM, and [H₂O₂] was varied between 0.3 and 40 mM. The four-fold oxidised product of the peroxidase reaction of cytc550, 3,3'-dimethoxy-4,4'-biphenylquinone [13] ($\epsilon_{470} = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$ [14]) was followed using a Shimadzu UVPC-2101PC spectrophotometer fitted with a thermostat. The obtained activity profiles were analysed as before [6]. Throughout, the reaction rate depended linearly on [H₂O₂]. For coherent graphical representation, the activities are expressed as the bimolecular rate constant of the H₂O₂–cytc550 reaction.

The electronic absorption spectra of cytc550 as a function of [detergent] were measured on the spectrophotometer described above. The fluorescence spectra of cytc550 were recorded on a Perkin-Elmer Luminescence Spectrometer LS-50B, with 290 nm as the excitation wavelength. Excitation and emission slit widths were 4 and 20 nm, respectively. A quartz cuvette of dimensions 0.2 by 1 cm was used.

The CMC of the detergents was determined using the method whereby NPN fluorescence was followed as a function of [detergent], as described by Brito and Vaz [15]. In the solutions, 0.61 or 4.4 μ M cytc550 and 10 mM guaiacol was included. NPN was excited at 356 nm, and excitation

and emission slit widths were 4 nm. A quartz cuvette of dimensions 0.2 cm $\times$ 1 cm was used. The presence of protein–detergent complexes did not influence NPN fluorescence (not shown) meaning that NPN was taken up by pure detergent micelles only.

The net overall charge of the protein was calculated as a function of pH, by making use of the amino acid composition [16] and their average pK_a values taken from Lehninger [17]. It is assumed that one of the two heme propionates titrates, with $pK_a = 6.2$, as with cytc550 from *P. denitrificans* [18]. The two cysteine residues do not titrate with pH, as they are covalently linked through thioether bonds to the heme. The iron-coordinating histidine and the N-terminus were assumed not to titrate either; the histidine is known to remain coordinated to the heme-iron over a broad pH range [18] and the N-terminus is pyroglutamated [16,19]. Eq. (1) was used to calculate the overall net charge, whereby b_i , K_a^{bi} , a_j , and K_a^{aj} are the number and acid constants of the i th base and j th acid, respectively.

$$\text{net charge} = \sum_{i=1}^n \frac{b_i \times [\text{H}^+]}{[\text{H}^+] + K_a^{bi}} - \sum_{j=1}^m \frac{a_j \times K_a^{aj}}{[\text{H}^+] + K_a^{aj}} \quad (1)$$

3. Results

3.1. Effect of SDS on cytc550 fluorescence, absorption spectrum and peroxidase activity

When SDS is added to cytc550, the tryptophan fluorescence increases (Fig. 1A). Tryptophan fluorescence quenching is usually ascribed to interactions with the heme, and so an increase in fluorescence can be ascribed to an increase in the tryptophan-heme distance and an overall expansion of the protein structure [20]. The fluorescence increase takes place between 0.25 and 1.0 mM SDS, and exhibits a sigmoidal shape, which implies cooperativity. Fig. 1B depicts the [SDS] dependence of the position of the main optical absorption (Soret band) of cytc550. A small blue shift occurs already at low (~0.1 mM) [SDS], but the major (blue) shift occurs between 0.25 and 0.45 mM SDS. This transition is followed by a red shift up to 0.7 mM SDS. In Fig. 1C, the peroxidase activity is shown versus [SDS]. At low (0.1 mM) [SDS] the activity is somewhat repressed, but increasing [SDS] induces a significant increase in the peroxidase activity of cytc550. The activity increase occurs between 0.25 and 0.7 mM SDS, after which the activity is repressed up to 1 mM SDS. The CMC was determined as 0.74 mM SDS (under the same conditions as the peroxidase activity experiments, except for H₂O₂). This is roughly concurrent with the major changes observed for the properties of cytc550. Above the CMC, preferential solubilisation of the reducing substrate guaiacol in pure SDS micelles may reduce its availability, and thus decrease the reaction rate. This is not the reason however, of the activity decrease above 0.7 mM

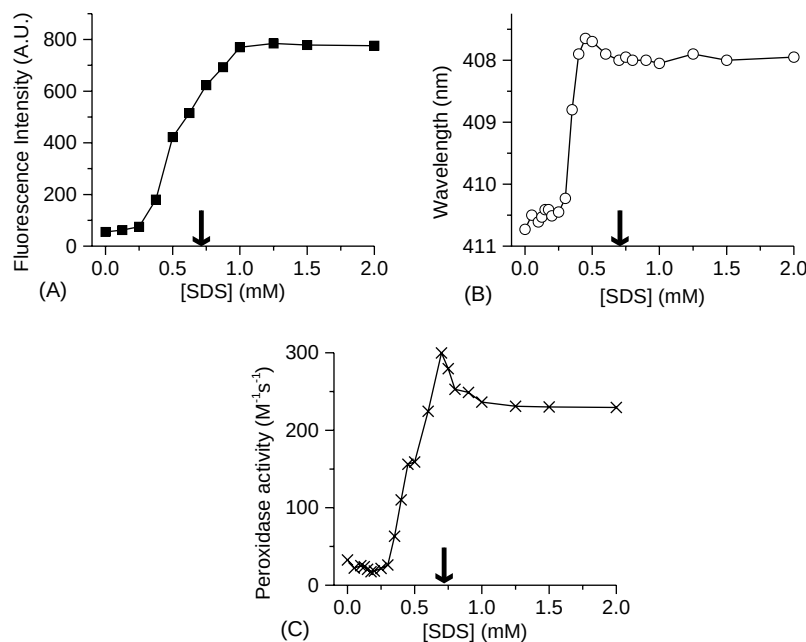


Fig. 1. Effect of SDS on selected properties of cytc550. (A) Tryptophan fluorescence. (B) Position of the main heme absorption band (Soret). (C) The peroxidase activity of cytc550. Experiments were performed in 100 mM sodium phosphate pH 8, with $0.61 \mu M$ cytc550, and for the peroxidase activity measurements, 10 mM guaiacol and between 3 and 15 mM H_2O_2 . The CMC of SDS was determined in the presence of 10 mM guaiacol and $0.61 \mu M$ cytc550, and its value is indicated in the plots by $\downarrow$. Indicated on the x-axis is the total [SDS].

SDS (above the CMC), because both in the presence and absence of SDS, the rate is governed by the bimolecular reaction between peroxide and cytc550 (not shown).

3.2. Dependence of SDS-induced peroxidase activity increase on buffer composition and counter-ions

The above-mentioned experiments were performed in the presence of 100 mM sodium phosphate, pH 8. To assess the influence of sodium phosphate, the ionic strength and nature of the SDS counter-ions, the peroxidase activity increase was determined in 10 mM Tris–HCl, pH 8, and also in this buffer supplemented with 100 mM LiCl. These results are shown in Fig. 2, together with the CMC-values determined under these conditions. The ability of SDS to affect peroxidase activity follows the same trend as the tendency to form micelles. The more solvophobic the detergent monomers (depending on the counter-ion), the lower the CMC, but also the peroxidase activity increase takes place at correspondingly lower [SDS]. Interestingly, under all conditions tested, the peroxidase activity reaches its maximal value at the CMC, after which a decrease in activity is seen. Note that the maximal activity as well as the amplitude of the decrease seems to depend on the buffer composition.

3.3. The pH dependence of the SDS-induced peroxidase activity increase

To study the effect of cytc550 surface charge on the binding of SDS, the peroxidase activity increase caused by SDS

was assessed as a function of pH. At neutral pH, cytc550 is a strongly charged protein, with a highly non-symmetrical distribution of its charged amino acid side chains on the surface. At the ‘front face’, around where the heme-edge is exposed to the solvent, cytc550 has a ring of basic lysine residues, while the ‘back face’ is studded with glutamic and aspartic acid residues and carries substantial negative charge. The iso-electric point of cytc550 is 4.6 [4]. The calculated overall charge, on basis of average pK_a values of its protonatable groups, is positive at pH 4, whereby the back face

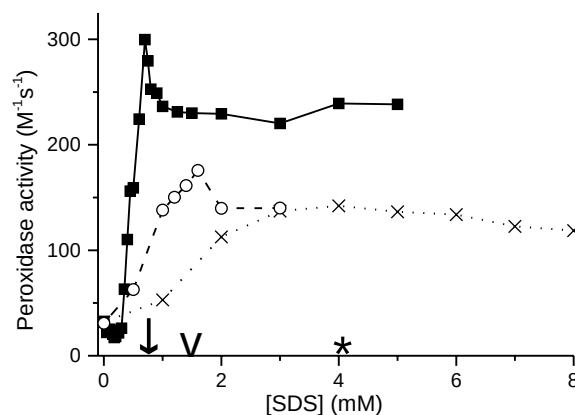


Fig. 2. Effect of the buffer composition on the SDS-dependence of the peroxidase activity of cytc550. Experiments were performed in 100 mM sodium phosphate pH 8 (filled squares, CMC indicated by $\downarrow$), 10 mM Tris–HCl pH 8 (crosses, CMC indicated by $\star$) and 10 mM Tris–HCl pH 8 supplemented with 100 mM LiCl (open circles, CMC indicated by $\downarrow$). Experimental details were as in Fig. 1C.

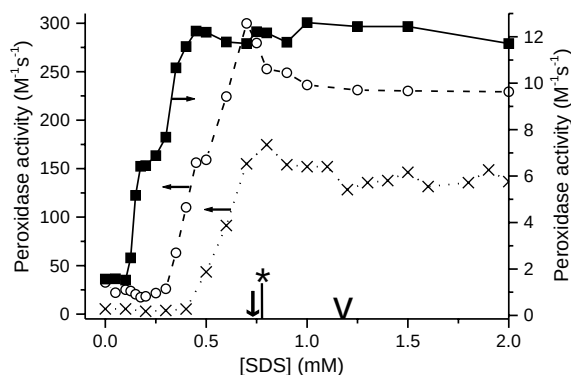


Fig. 3. Peroxidase activity of cytc550 as a function of total added SDS at pH 4 (filled squares, CMC indicated by $\vee$), pH 8 (open circles, CMC indicated by $\downarrow$) and pH 11 (crosses, CMC indicated by $\star$). Note that the left y-axis refers to the data at pH 8 and 11, and the right y-axis refers to the data at pH 4. In all cases [cytc550] was $0.61 \mu\text{M}$, [guaiacol] was 10 mM , $[\text{H}_2\text{O}_2]$ between 2 and 40 mM , and [phosphate] was 100 mM .

of cytc550 will be partly neutralised. Conversely, at pH 11, the positively charged patch around the exposed heme-edge is partly neutralised. At pH 8, both patches are charged.

In Fig. 3, the peroxidase activity of cytc550 is plotted as a function of [SDS] at pH 4, 8 and 11, with the CMC of SDS under the conditions of each experiment also indicated. The curves at pH 8 and 11 are similar, albeit that the mid-point of the transition is 0.48 and 0.6 mM at pH 8 and 11, respectively. The affinity of SDS to bind cytc550 is highest at pH 4, where the transitional midpoint is at $\sim 0.225 \text{ mM}$ SDS. Interestingly the peroxidase activity increase is complete well before the CMC of SDS at pH 4 and also the peroxidase activity does not exhibit a decrease after reaching its maximum.

The enhanced affinity of SDS for cytc550 at acid pH suggests that binding of SDS to cytc550 is based on the attraction between the negatively charged SDS molecules and positive charges on the protein. It is interesting that going from pH 8 to 4, cytc550 is expected to have a smaller number of negative charges (23 and 11 at pH 8 and 4, respectively), while the amount of positive charges remains essentially unaltered (~ 16). Neutralisation of the negatively charged residues apparently enhances the binding affinity of SDS for cytc550. Going from pH 8 to 11, cytc550 loses about 11 of its positive charges. Although this does affect the binding affinity somewhat, it is not very much poorer at pH 11 than at pH 8.

3.4. The effect of non-ionic and zwitterionic detergents on the peroxidase activity of cytc550

The effect of several non-ionic and zwitterionic detergents and amphiphiles on the peroxidase activity of cytc550 was assessed (pH 8). No effect on the activity is observed with sodium cholate, sodium desoxycholate, *N*-dodecyl-*N,N*-dimethylammonio-3-propane sulfonate, *N,N*-dimethyl dodecylamine-*N*-oxide, sodium bis-(2-ethylhexyl)

sulfosuccinate and Triton X-100 (not shown). This is not surprising, as non-ionic and zwitterionic detergents tend not to disturb protein structure [21]. Their obvious lack of interaction also with cytc550, corroborates that cytc550 unfolding is triggered by or depends on the attraction between opposite charges on protein and detergent.

3.5. The effect of detergent chainlength and headgroup charge on the peroxidase activity of cytc550

The effect of hexadecyltrimethylammonium bromide (HTAB), a positively charged detergent with a tail 16 carbon atoms long, on the peroxidase activity of cytc550 is shown in Fig. 4 at pH 4, 8 and 11, respectively, together with the experimentally determined CMC values. HTAB influences the peroxidase activity of cytc550 in a similar fashion as seen with SDS (see above). A major increase in activity is followed by a decrease. The peroxidase activity increase is roughly concurrent with the CMC, except for the peroxidase increase at pH 11. The fact that the HTAB–cytc550 complex saturates before the CMC under alkaline conditions is in interesting contrast to the case of the anionic detergent SDS, which complexes cytc550 well before the CMC under acidic conditions. The affinity of HTAB to bind cytc550 thus increases with increasing pH, which is probably related to the increasing overall negative charge of cytc550. In addition, the decreasing hydrophilicity of HTAB with increasing pH (as seen in the decreasing CMC) probably also plays a role in the enhanced affinity. The latter is probably caused by an increased population of doubly or triply charged phosphate ions in the solution, as these are effective counter-ions for HTAB [22].

The peroxidase activity increase induced by dodecyltrimethylammonium bromide (DTAB) (with a chain length of 12 carbon atoms) is shown in Fig. 5 at three pH values together with the experimentally determined CMC values. At pH 4 and 11, a steep increase in activity is observed, that subsequently decreases after passing the CMC. At pH 8, the curve appears quite different: the peroxidase activity increase occurs over a much broader DTAB concentration than at lower and at higher pH, and no decrease of peroxidase above the CMC of DTAB is seen.

The effect of tetradecyltrimethylammonium bromide (TTAB, with a chain length of 14 carbon atoms) on the peroxidase activity of cytc550 at pH 8 is shown in Fig. 6. The activity curve shows the same trend as observed before, i.e. a significant, sigmoidal increase in the peroxidase activity with increasing [detergent] up to the CMC. In the case of TTAB, no decrease in activity is seen above the CMC.

The affinity to bind cytc550 clearly depends strongly on the length of the aliphatic tail of the three tested cationic detergents. The affinity is higher when the tail is longer. In most cases, the activity increase is more or less concurrent with the CMC, placing a clear link between the propensity of the detergent to form micelles and its propensity to form a complex with cytc550.

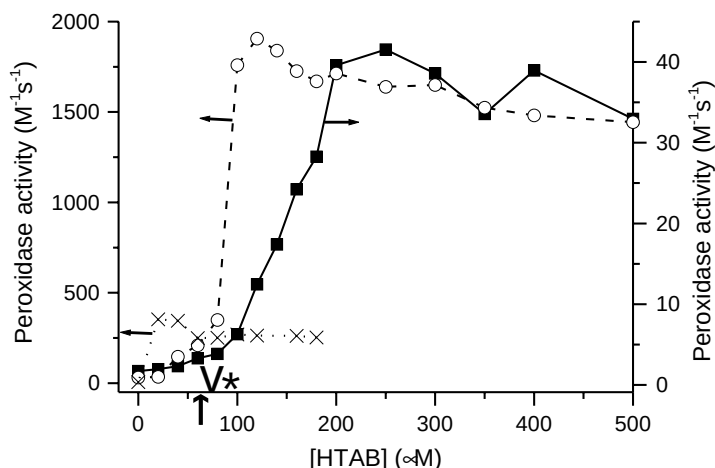


Fig. 4. Peroxidase activity of cytc550 as a function of total added HTAB at pH 4 (filled squares, CMC indicated by ★), pH 8 (open circles, CMC indicated by ∇) and pH 11 (crosses, CMC indicated by ↑). Note that the left y-axis refers to the data at pH 8 and 11, and the right y-axis refers to the data at pH 4. In all cases [cytc550] was 0.61 μ M, [guaiacol] was 10 mM, [H₂O₂] between 0.6 and 30 mM, and [phosphate] was 100 mM.

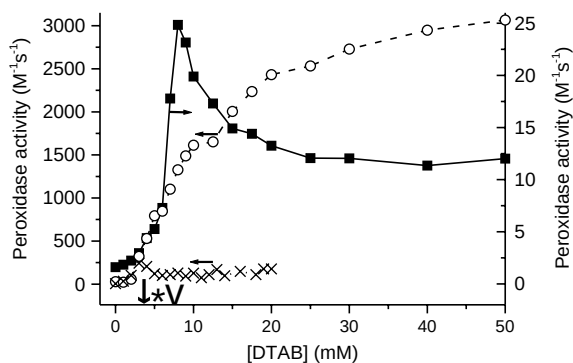


Fig. 5. Peroxidase activity of cytc550 as a function of total added DTAB at pH 4 (filled squares, CMC indicated by ★), pH 8 (open circles, CMC indicated by ∇) and pH 11 (crosses, CMC indicated by ↓). Note that the left y-axis refers to the data at pH 8 and 11, and the right y-axis refers to the data at pH 4. In all cases [cytc550] was 0.61 μ M, [guaiacol] was 10 mM, [H₂O₂] between 0.3 and 30 mM, and [phosphate] was 100 mM.

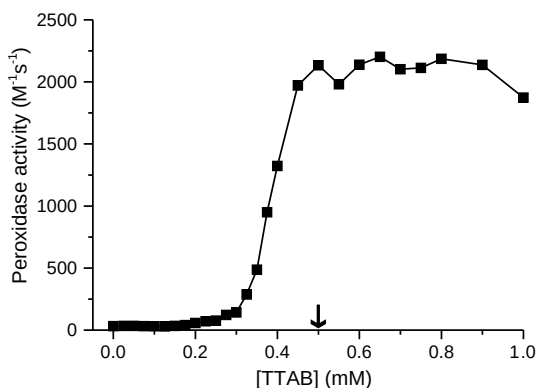


Fig. 6. Peroxidase activity of cytc550 as a function of total added TTAB in 100 mM sodium phosphate pH 8. [Cytc550] was 0.61 μ M, [guaiacol] was 10 mM, [H₂O₂] between 0.6 and 30 mM. The ↓ indicates the CMC determined for TTAB.

3.6. Effect of protein concentration on the detergent-induced peroxidase activity increase

The importance of protein concentration was assessed for the effects of SDS and HTAB on the peroxidase activity of cytc550 (Fig. 7). The SDS-binding curve at 4.4 μ M cytc550 is distinct from that at 0.61 μ M cytc550 (Fig. 7). The affinity appears lower, and also the typical sharp maximum around the CMC is absent at higher [cytc550]. The apparent decrease in affinity is simply related to the fact that the amount of unbound detergent is smaller when the protein concentration is higher (note that on the x-axis in Fig. 7, the total (bound and unbound) [SDS] is plotted). Accordingly, the transition seems slightly less steep at 4.4 μ M cytc550. Moreover, the CMC was determined to be $\sim$ 65 μ M higher at 4.4 μ M cytc550. The protein concentration dependence of the binding of HTAB to cytc550 (Fig. 7) is comparable to that just discussed for the case of SDS. The CMC of HTAB was determined to be $\sim$ 85 μ M higher in the presence of 4.4 μ M cytc550.

Assuming that the difference in CMC is caused by the decreased concentration of free detergent due to the binding of detergent to extra 3.79 μ M protein, it is easily calculated that cytc550 binds $85/3.79 = 23$ monomers HTAB and $65/3.79 = 17$ monomers SDS, respectively. Despite the crudeness of this approach to determine the number of detergent molecules bound by cytc550, the apparent number of detergent monomers in the protein–detergent complex relates well to the amount previously observed to bind ($\sim$ 20) to the closely related horse heart cytochrome *c* [23,24]. Interestingly the estimated number of 23 HTAB molecules bound to cytc550 is the same as the number of negative charges at pH 8. Likewise, the estimated number of SDS molecules bound, 17, corresponds remarkably well to the 16 positive charges that are present on cytc550 at pH 8. Although this

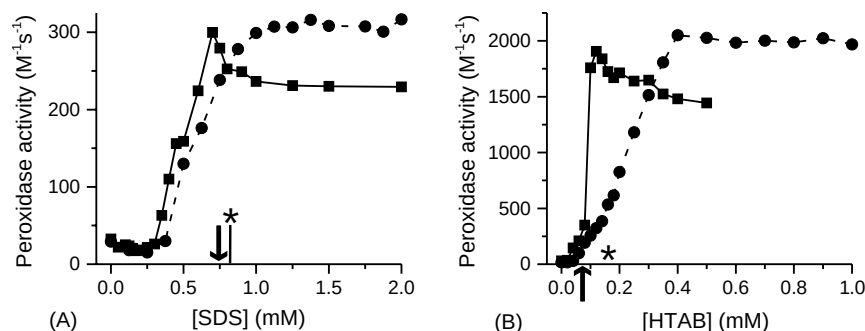


Fig. 7. Protein concentration dependence of the detergent-induced peroxidase activity of cytc550. (A) Peroxidase activity of 0.61 μM cytc550 (squares) and 4.4 μM cytc550 (circles) as a function of total added SDS. (B) Peroxidase activity 0.61 μM cytc550 (squares) and 4.4 μM cytc550 (circles) as a function of total added HTAB. Each experiment was performed in 100 mM sodium phosphate pH 8, using 10 mM guaiacol and between 0.75 and 3 mM H_2O_2 . The $\downarrow$ and $\star$ indicate the CMC, whereby the $\star$ refers to the CMC in the presence of 4.4 μM cytc550.

agreement in numbers may be entirely coincidental, it enforces the idea that detergent–protein complex formation is driven by electrostatic interactions between opposite charges on the protein and the detergent headgroups.

4. Discussion

It is well known that ionic detergents induce structural perturbations in proteins [21,25,26]. The properties of the resultant complexes and the mode of complex formation have been intensively studied ([27–29] and references therein). Nevertheless, much remains unclear, which is related to the heterogeneity of protein–detergent complexes and to the fact that details of the interaction depend strongly on the type of detergent chosen and the properties of the protein studied. In some instances, *c*-type cytochromes have been used to study protein–detergent interactions [23,24,30–35]. These proteins have intrinsic advantages for such studies as they have a covalently bound heme-cofactor, which provides useful spectroscopic handles to monitor complex formation with [18,35]. Moreover, the heme-cofactor has an intrinsic peroxidase activity, which can be exploited to study cytochrome unfolding [8] as we have done here.

As it turns out, the properties of cytc550 that are related to the heme-cofactor display a more complex dependence on detergent concentration than the protein fluorescence, which is more related to the overall properties of the protein (Fig. 1). Whereas with increasing [SDS], the fluorescence shows a simple S-shaped increase, the major heme absorption band (Soret band) and the peroxidase activity exhibit a more complex dependence on [SDS]. The fluorescence increase is compatible with an increase in the average size of cytc550 upon binding of SDS (i.e. the protein unfolds). An increased average distance between the tryptophan fluorophores and the covalently bound heme leads to a less efficient energy transfer between the tryptophans and the heme, and consequently the fluorescence increases [20].

The position of the Soret band as a function of [SDS] however, is already affected at significantly lower concentrations

(at 0.1 mM SDS at pH 8). This is concurrent with a small decrease in peroxidase activity, at least with anionic detergent, which we ascribe to specific binding of SDS on the positively charged front face of cytc550. The main cooperative transition, which is simply sigmoidal when followed by fluorescence, displays a distinct maximum in the shift of Soret band position as well as in the peroxidase activity. This maximum occurs before SDS-binding reaches a plateau, as signified by the continuing fluorescence increase with increasing [SDS] (cf. Fig. 1A–C). Moreover, the maximum in the Soret band shift occurs at a different position than the maximum observed in the peroxidase activity. Despite this apparent disparity, both effects may relate to the same case but reflect differences in the size of the observed effect in initial and later stages of SDS-binding. The initial blue shift of the Soret band and the initial increase in activity probably reflect an increased accessibility of the heme cavity for water molecules and for the co-substrate, H_2O_2 , respectively. In addition, the increased dissociation of the axial methionine heme ligand [23,32,35] (which blocks access of peroxide to the heme iron) may play a role.

The partial reversal of the Soret band shift and the activity increase at still higher SDS concentrations is interpreted as signalling a decreased accessibility of the heme cavity and a concomitant change again in the polarity of the medium around the heme. This may also affect (increase) the pK_a of the hydrogen peroxide, which would lower the effective concentration of the peroxide anion (the reactive species) [6–8].

This partial reversal of the peroxidase activity increase is also observed with cationic detergents (Figs. 4–6). The extent of the decrease, if at all, depends very much on the nature of the detergent and buffer composition. Thus, in some cases, the microenvironment of the heme in the protein–detergent complexes is changed significantly upon reaching full saturation with detergent. This is best illustrated by the behaviour of DTAB (Fig. 5). At pH 4 and 11, a significant activity decrease occurs at the CMC, while at pH 8 the exact opposite is observed. Note that when a higher cytc550 concentration is used, the decrease is also less pronounced

(HTAB, Fig. 7) or absent (SDS, Fig. 7). The reason for this may be that at higher protein concentrations, the free detergent monomer concentration required for full saturation of the protein is not reached.

In general, the peroxidase activity as a function of the detergent concentration always increases at detergent levels close to the CMC. Detergents with longer aliphatic tails, having a stronger tendency to form micelles, affect cytc550 at lower concentrations (the HTAB–TTAB–DTAB series). Buffer compositions that promote micelle formation, i.e. having higher ionic strength or containing strong counter-ions cause the peroxidase activity to increase at lower detergent levels (e.g. see Fig. 2). Although there are some interesting exceptions to this general observation (vide infra), there is a clear connection between micelle formation and the formation of a protein–detergent complex. In most cases, detergent–protein complexes are formed or are still in the transition phase just below the CMC.

There is also a correlation between detergent charge and protein charge, implying a role for electrostatics in the association between detergent and cytc550. This is clearly exemplified by the failure of non-ionic and zwitterionic detergents to increase the peroxidase activity of cytc550. Also, there is the interesting observation that when the protein contains a significant amount of charges opposite to that of the detergent headgroups, complex formation can occur at significantly lower [detergent] than the CMC. This includes two of the above-mentioned exceptions, namely the saturation of the cytc550–SDS complex at low pH (Fig. 3) and the saturation of the cytc550–HTAB complex at high pH (Fig. 4). Under these conditions, complex formation is apparently so favourable as a result of electrostatic forces, that protein–detergent complexes are formed at significantly lower detergent concentration than pure detergent micelles.

There appears to be a significant difference in the ability of the anionic SDS and the quaternary ammonium-based detergents to enhance the peroxidase activity of cytc550. The peroxidase increase caused by SDS is moderate except at alkaline pH. The total increase in activity for each detergent with respect to the activity under native conditions is shown in Table 1, together with the total increase observed when cytc550 is unfolded by guanidinium hydrochloride. The activity under native conditions is inhibited by the methionine iron ligand and the increase in activity by addition of denaturant reflects the propensity of the denaturant to weaken the methionine-iron bond, although the structure and availability of inhibiting ligands in the unfolded protein also plays a role [7,8]. It emerges that guanidinium hydrochloride is much more powerful than the detergents studied here to induce peroxidase activity in cytc550. This is not surprising, considering that guanidinium hydrochloride tends to cause complete unfolding in proteins as will be discussed below. The activity under native conditions, against which the activity increase is measured, is much lower at pH 8 than at pH 4 (corrected for peroxide anion as the reactive species). This is related to the strength of the methionine-iron bond, which

Table 1

Maximal increase in peroxidase activity relative to the native state, for the different detergents used in this study, and including guanidinium hydrochloride

Unfolding agent	Peroxidase increase relative to native conditions (times)		
	pH 4	pH 8	pH 11
SDS	8	9	32
HTAB	26	60	64
TTAB	n.d. ^a	69	n.d. ^a
DTAB	16	>95	45
Guanidinium hydrochloride	490 ^b	1800 ^b	n.d. ^a
Peroxidase activity ($M^{-1} s^{-1}$)			
None ^c	1.8×10^4	63.8	5.3

^a Not determined.

^b Data from [8].

^c Peroxidase activity of cytc550 in the absence of detergent, corrected for the concentration of HO_2^- in solution, using $pK_{a,app}(H_2O_2) = 8.04$ [8].

is weaker at pH 4 [8]. Consequently, the relative activity increase at pH 8 upon denaturation is larger than at pH 4. At alkaline pH, the activity increase tends to be even higher because the methionine ligand is replaced by a lysine ($pK_a \sim 11.2$) [36], which inhibits the peroxidase activity even more under non-denaturing conditions [8]. These considerations also hold for the detergent-induced activity increase as summarised in Table 1.

Guanidinium hydrochloride and similar chaotropic agents tend to cause complete unfolding, whereas detergents induce partial unfolding and often a significant degree of secondary structure remains in the protein–detergent complexes [27,26,35]. It has been demonstrated by circular dichroism studies that only about 25% of the secondary structure is lost upon addition of SDS to horse heart cytochrome *c* [33,35], which is a closely related protein to cytc550 [16,18]. In contrast, all secondary structure is lost in the presence of guanidinium hydrochloride [35]. Although the tertiary structure is probably severely perturbed in the detergent-complexed cytc550, by comparison to horse heart cytochrome *c* it is thus likely that a significant degree of secondary structure remains in the fully complexed protein, rendering it fairly compact with respect to the guanidinium hydrochloride-unfolded form. In addition, in the added presence of multiple detergent molecules, it can be envisaged that the heme-iron is much more shielded from the oxidising substrate in the protein–detergent complexes than in guanidinium hydrochloride-unfolded cytc550, hence resulting in a less large increase in the peroxidase activity. In addition, as discussed above, the polarity of the heme environment may be an important factor, as it is the peroxide anion that is the oxidising substrate. Full complexation of cytc550 by detergent decreases the polarity around the catalytic heme-iron centre, which probably results in a less high effective concentration of peroxide anion by an increased pK_a of hydrogen peroxide deprotonation.

Whereas the factors have been resolved that influence the affinity of the detergent for cytc550, it is yet unclear what determines the extent of the increase in peroxidase activity by the action of detergents. This appears to be related to factors such as crowdedness and polarity of the heme-environment in the fully saturated detergent–cytc550 complexes.

This study was undertaken to explore the potential of detergents to enhance the catalytic activity of cytc550 for use as a ‘green’ catalyst in oxidative chemistry. It appears that cationic detergents have considerable capability to enhance the peroxidase activity of cytc550. Nevertheless, the activity of detergent-complexed cytc550 as compared to true peroxidase enzymes is still low (at least 10,000 times lower). Exceptional is that cytc550 becomes activated in the presence of detergents, rather than inactivated like most proteins. This peculiar property of cytc550 makes it an attractive candidate as an oxidative catalyst for use in the presence of detergents. Its robustness at extremes of pH and heat provide additional advantages of cytc550 over conventional oxidising enzymes.

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