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Peroxidase Activity as a Tool for Studying the Folding of *c*-Type Cytochromes†

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ABSTRACT: The peroxidase activity of *c*-type cytochromes increases substantially by unfolding. This phenomenon was used to study the equilibrium unfolding of ferricytochrome *c*. The peroxidase activity is already enhanced at low denaturant concentrations. The lowest free energy folding intermediate is easily detected by this method, while it is invisible using fluorescence or optical spectroscopy. The free energy difference between this folding intermediate and the native state depends on the strength of the sixth ligand of the heme-iron and the increase in peroxidase activity upon unfolding is shown to be a sensitive indicator of the strength of this ligand. Under fully denaturing conditions, the peroxidase activity is inhibited by protein-based ligands. It is shown that at least three different ligand groups can be responsible for this inhibition, and that at neutral or alkaline pH, the predominant ligand is not histidine. The use of peroxidase activity assays as a method to study the unfolding of cytochrome *c* is evaluated.

Cytochrome *c* (cytc)[1] has been used over the past years as a paradigm for understanding protein folding. Many *c*-type cytochromes are small and highly soluble proteins both in their folded and unfolded states, and the prosthetic group, a heme, provides a multitude of spectroscopic parameters. This allows for a detailed study of folding, because the various (spectroscopic) methods sense different features of the protein structure and hence of its state of folding. Equilibrium-unfolding curves monitored by different methods tend to give different outcomes (*1*) in terms of the unfolding free energy ΔG, and the *m*-value, which is a measure of the cooperativity of the unfolding. This indicates that although each curve may be fitted to a two-state model of unfolding, there actually are one or more intermediate states between the native and fully unfolded states (*2*). The presence of some of these intermediates may escape detection when techniques such as fluorescence, UV/vis or CD spectroscopy are used, causing confusion in the analysis of the unfolding data (*3*, *4*). Moreover, because unfolding intermediates are often so poorly populated, it is difficult to study their properties.

Recently it was discovered that unfolding the Class I cytc (cytc-550) from *Paracoccus versutus* has a spectacular effect on the intrinsic peroxidase activity of this protein (*5*). This prompted us to explore the use of peroxidase activity measurements to study cytc unfolding. In general, *c*-type cytochromes catalyze the oxidation of reducing substrates in the presence of H_2O_2. Although details are unclear, after binding to the heme center, hydrogen peroxide is probably cleaved heterolytically, yielding high-valent protein intermediates capable of substrate oxidation (*6*). In contrast to true peroxidase enzymes, cytc-550 requires the deprotonated form of H_2O_2 to react. This is because cytc-550 lacks the His residue near the heme-iron that acts as a general acid−base catalyst in peroxidase enzymes (*6*). Moreover, peroxidase activity requires a free coordination position on the heme-iron. Hence cytc-550 displays extremely low peroxidase activity, compared not only to true heme peroxidases (*7*) but also to nonperoxidase pentacoordinated heme proteins such as hemo- and myoglobin (*8*).

The native, six-coordinate state has no peroxidase activity and only the small population of (partially) unfolded, five coordinate cytc550 acts as a peroxidase (*5*). The activity increase upon unfolding can be attributed to loss of the native Met ligand, and increased access to the heme-iron. Interestingly, intermediates in cytc unfolding are generally perturbed in heme-iron coordination and therefore may possess peroxidase activity. This makes the approach to study cytc unfolding using a peroxidase activity assay especially sensitive under conditions favoring the folded state and allows for the study of the lowest free energy unfolding intermediates.

In this paper, the peroxidase activity of four different *c*-type cytochromes as a function of their conformation is reported. It is shown that the peroxidase activity increase that is brought about by the addition of denaturant is related to partial unfolding. Analysis of the unfolding curves indicates a transition to an unfolding intermediate of similar free energy as established earlier using NMR techniques (*9*, *10*). The stability of the lowest free energy intermediate is assessed as a function of pH and heme-iron ligation. The total increase in peroxidase activity upon unfolding, as related

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[1] Abbreviations: cytc, cytochrome *c*; cytc-550, cytochrome *c*-550 from *P. versutus*; M100K cytc-550, the Met100Lys mutant of cytc-550; iso-1-cytc C102T, the Cys102Thr mutant of iso-1-cytochrome *c* from yeast; cytc-551, cytochrome *c*-551 from *Ps. aeruginosa*; UV/vis, ultraviolet−visible; NMR, nuclear magnetic resonance; CD, circular dichroism; MP-8, microperoxidase-8; Gdn·HCl, guanidinium hydrochloride; wt, wild type.

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to the activity in absence of denaturant, is shown to be a sensitive indicator of the strength of the sixth heme-iron ligand. Thus, the magnitude of the activity increase provides information on the state of cytc in the absence of denaturant. Characterization of the fully unfolded cytochromes demonstrates that the unfolded protein matrix is of no influence on this activity, except under neutral and alkaline conditions, where protein-based ligands inhibit the reaction. Inhibition by His residues can be distinguished from inhibition by other, protein-based ligands. Finally, the usefulness of applying peroxidase activity studies on equilibrium unfolding of *c*-type cytochromes is discussed.

EXPERIMENTAL PROCEDURES

P. versutus cytc-550 was produced in *Paracoccus denitrificans* strain 2131 and isolated as described (*6*). Mrs. Ing. Gertrüd C. M. Warmerdam kindly supplied *P. versutus* M100K cytc-550, which was isolated as described (*11*). Cytc-551 was a kind gift of Drs. Ellen C. de Waal and Dr Erik Vijgenboom. It was produced in *Pseudomonas aeruginosa* strain PAO1, as described (*12*), and was isolated using a slightly modified protocol used previously to isolate *Ps. aeruginosa* azurin heterologously expressed in *Escherichia coli* (*13*). Dr Jonathan A. R. Worrall generously provided *Saccharomyces cerevisiae* iso-1-cytc C102T, produced in *E. coli* and isolated as described (*14*, *15*). Horse heart cytc (Type VI, Sigma) was subjected to Sephadex Superose-200 gel filtration before use. MP-8 was a kind gift of Ms. Mariel Boersma and Prof. Dr Ivonne M. C. M. Rietjens (Wageningen University), and was prepared as described (*16*). Throughout, the cytochromes *c* were used in their ferric form.

Chemicals were of the highest grade commercially available, and dissolved in de-ionized water (Milli-Q). Guanidinium hydrochloride (Gdn·HCl, Biochemika grade, Fluka) was dissolved to 8 M and this stock solution was filtered over 0.45 μM HV Durapore filters (Millipore) before use. The solutions were buffered with 0.1 M sodium phosphate. The pH of each solution was measured separately with a Corning pH pencil gel combo-electrode calibrated with IUPAC standard buffers (Radiometer Analytical, France). The cytc/Gdn·HCl mixtures had been equilibrated overnight before the measurements. All experiments were performed at 298 K.

Peroxidase activity was assayed using hydrogen peroxide and guaiacol (*o*-methoxyphenol, Sigma). The reaction was initiated by mixing a volume of cytc/Gdn·HCl with an equal volume of freshly prepared H_2O_2/guaiacol/Gdn·HCl. The formation of product, tetraguaiacol ($\epsilon_{470} = 26.6\ mM^{-1}\ cm^{-1}$), was followed using a Shimadzu UVPC-2101PC spectrophotometer fitted with a thermostat. The obtained activity profiles were analyzed as before (*6*). [Guaiacol] was 10 mM, [cytc] was between 0.5 and 3 μM, and [H_2O_2] between 0.1 mM and 100 mM. In all assays, the reaction rate depended in a bimolecular fashion on [cytc] and [H_2O_2]. For coherent graphical representation, the activities were normalized to 1 μM cytc and 1 mM H_2O_2, and it was taken into account that tetra-guaiacol is the product of four oxidation reactions (*17*). Optical spectra were recorded on the same instrument mentioned above. Fluorescence spectra were recorded on a Perkin-Elmer Luminescence Spectrometer LS-50B, with 290 and 359 nm as excitation and emission wavelengths, respectively.

Unfolding curves were assessed by the two-state model of unfolding assuming linear baselines, according to the method of Santoro and Bolen (*18*). Equation 1 was used to fit the data:

$$\text{observable} = \frac{a + b[\text{Gdn·HCl}] + (c + d[\text{Gdn·HCl}])\exp\dfrac{-\Delta G + m[\text{Gdn·HCl}]}{RT}}{1 + \exp\left(\dfrac{-\Delta G + m[\text{Gdn·HCl}]}{\text{RT}}\right)} \quad (1)$$

Herein, *a* and *c* correspond to the values of native and fully unfolded protein at zero denaturant concentration, respectively, and *b* and *d* to their respective dependence on [Gdn·HCl] (pre- and posttransitional baselines). ΔG is the Gibbs free energy of unfolding in absence of denaturant; *m* represents the dependence of the unfolding free energy on [Gdn·HCl]. In case of peroxidase activity measurements, the following modification was applied. The native state is assumed fully inactive, because in the native state the heme-iron is six coordinate and thus cannot react with H_2O_2. Since the native state is fully inactive, its activity cannot change linearly with increasing [Gdn·HCl], and thus the pretransitional baseline was set to zero in the fits. Thus the terms *a* and *b* in eq 1 were set to zero in the fitting procedure. All fits (nonlinear least-squares fitting) were generated using the algorithm of Levenberg and Marquardt, and done in Origin software, version 6.0 (Microcal Software, Northampton, MA).

RESULTS AND DISCUSSION

Choice of Cytochromes c. The following *c*-type cytochromes were studied: horse heart cytc, *S. cerevisiae* iso-1-cytc C102T, *Ps. aeruginosa* cytc-551, and *P. versutus* cytc-550. All four are Class I cytochromes, which have relatively low sequence homology, but are very similar in structure (*19*−*21*). Main differences involve size and the presence or absence of certain loops in the variable regions of this structural family (see Figure 1) (*20*).

These proteins were selected because, except for cytc-550, their folding has been well studied, which facilitates the interpretation of the unfolding data (*22*−*24*). *P. versutus* cytc-550 was included in this study because its peroxidase activity under native and denaturing conditions has been characterized (*5*, *6*). In addition, M100K cytc-550 was studied, permitting a direct comparison of the effect of the axial ligation on the unfolding properties. M100K cytc-550 is a site-directed mutant of *P. versutus* cytc-550 in which Lys replaces the wild-type axial iron ligand Met (*11*). Finally, microperoxidase-8 (MP-8) was studied. MP-8 is the product of extensive proteolytic digestion of horse heart cytc (*25*). It consists of heme-*c* to which a short stretch of amino acids is covalently bound, including the native His ligand. Its coordination chemistry and peroxidase activity are well characterized (*17*, *26*−*28*).

Unfolding Induces Peroxidase Activity in Horse Heart Cytochrome c. Unfolding of horse heart cytc by Gdn·HCl can be observed by methods such as fluorescence (Figure 2A), Soret band shift (Figure 2B) and decrease in absorption at 695 nm (Figure 2C). Addition of Gdn·HCl also affects the peroxidase activity of horse heart cytc (Figure 2D, open

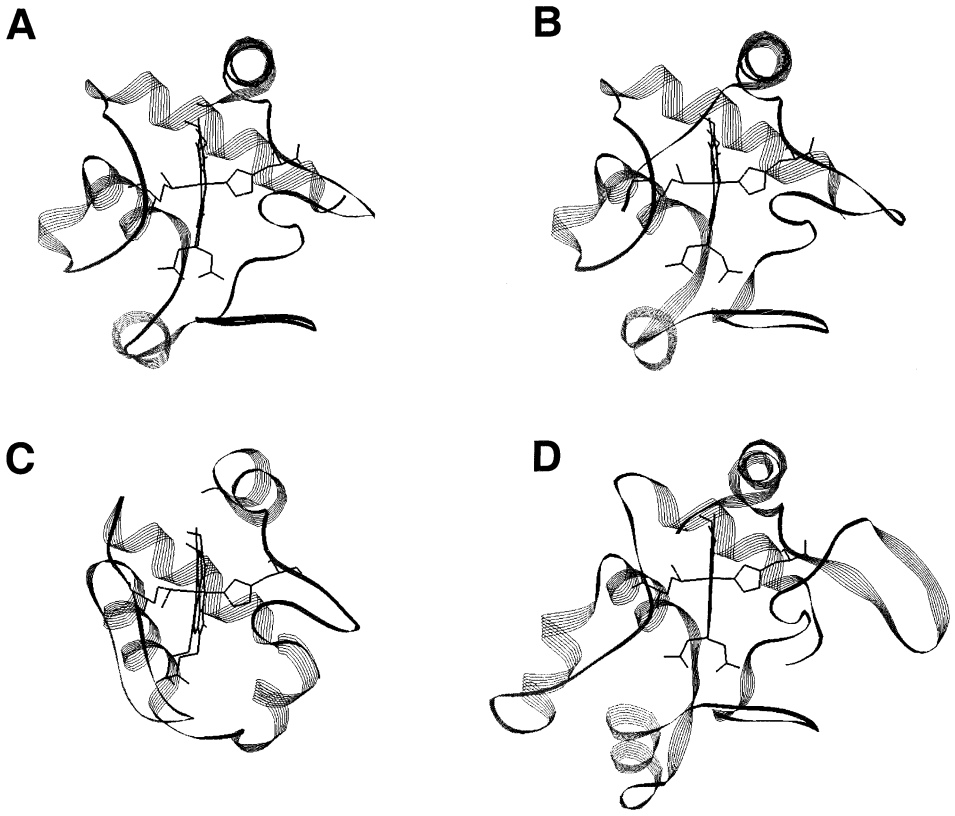

FIGURE 1: Schematic representation of the crystal structures of the *c*-type cytochromes used in this study. Panels A−D respectively depict horse heart cytc (*54*), yeast iso-1-cytc C102T (*55*), *Ps. aeruginosa* cytc-551 (*56*), and *P. denitrificans* cytc-550 (*57*). Although no structure has yet been solved for *P. versutus* cytc-550, it is thought to be very similar to the highly homologous cytc-550 from *P. denitrificans* (*58*).

circles). The sigmoidal dependence of the peroxidase activity on [Gdn·HCl] corresponds to an unfolding transition leading to a heme iron that is more accessible to peroxide. The experimental data in Figure 2 were fitted (solid lines) to the two-state model of unfolding (*18*). The apparent free energy change and *m*-value (Table 1) obtained from fitting the transition measured by peroxidase activity are much lower than measured by fluorescence or either of the optical techniques. This indicates that the peroxidase activity assays detect a low free energy unfolding intermediate that remains obscure in the optical and fluorescence measurements (*2*, *29*). If the unfolding were strictly two-state, then at zero [Gdn·HCl] the fraction of fully unfolded horse heart cytc is $\sim 2.7 \times 10^{-6}$, based on $\Delta G_{unf} = 7.6$ kcal/mol (the average of the spectroscopic measurements, Table 1). However, going from 0 to 6.0 M Gdn·HCl, the activity only increases ~375 times. Thus at zero [Gdn·HCl], a substantial fraction of horse heart cytc is in a form that is peroxidase-active (i.e. nonnative), but that is not the fully unfolded species either. The contribution of this unfolding intermediate to the peroxidase activity at lower Gdn·HCl concentrations is obvious when the unfolding curves measured by fluorescence and peroxidase activity are overlaid (Figure 2A). The clear observation of the intermediate is probably due to the large difference in peroxidase activity between the intermediate and the native state (which has zero peroxidase activity).

The 695-nm band is indicative of Met-ligation (*20*) and its loss is known to precede slightly the global unfolding in *c*-type cytochromes (*30*, *31*). Kinetic studies indicate that Met-iron coordination is the final step in cytc folding (*22*). Yet, unfolding measured by peroxidase activity indicates an unfolding transition of even lower free energy. This is surprising because it is expected that the peroxidase activity increase is related directly to increased accessibility of the heme-iron, following release of the Met ligand. It may be argued that the transition measured by peroxidase activity is shifted with respect to the transition monitored at 695-nm, as peroxide-binding affects the Met-Fe binding equilibrium. This is unlikely however, because the peroxide concentrations used in these experiments are far below the K_M of this substrate, both at zero and high [Gdn·HCl]. A more likely reason is that the 695-nm experiments have low sensitivity to small amounts of (partially) unfolded species. As a consequence, in the 695-nm measurements, the presence of unfolding intermediates at low [denaturant] eludes detection, and the two-state fit is biased toward the calculation of the global unfolding transition. On the other hand, the peroxidase activity is very sensitive to non-native species at low denaturant concentrations, as the native state has zero peroxidase activity. The unfolding curve measured by peroxidase activity (Figure 2D, Table 1) is thus much more representative of the transition to the lowest-lying free energy intermediate. This is substantiated by peroxidase activity measurements at pH 7.0 and pH 8.0 (vide infra). These indicate a lowest lying unfolding intermediate of free energy comparable to that observed using isotope exchange NMR (*9*), and in a recent NMR study following heme resonances as a function of denaturant (*10*).

Whereas the peroxidase activity of horse heart cytc and other *c*-type cytochromes (vide infra) increases in a sigmoidal fashion with increasing [Gdn·HCl], the peroxidase activity of MP-8 shows a linear increase (Figure 2D, crosses), in

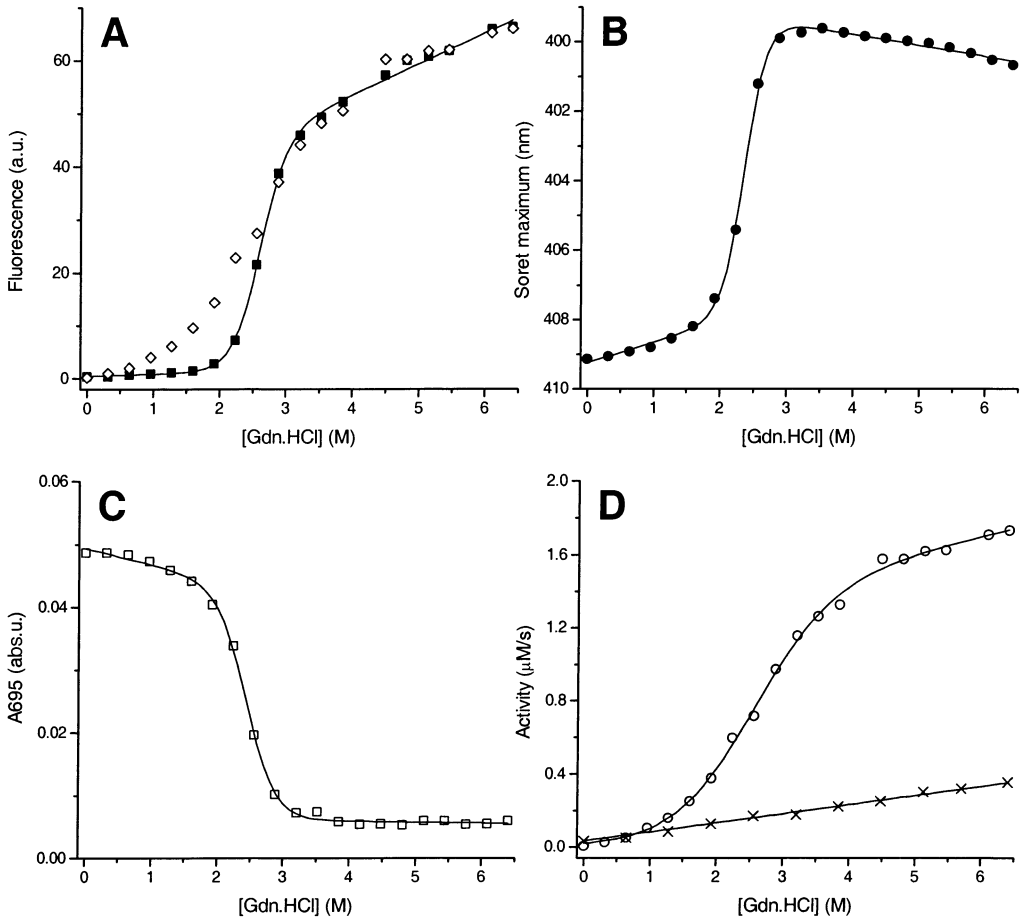

FIGURE 2: Equilibrium unfolding of horse heart cytc by Gdn·HCl at pH 4.5 (298 K) followed by tryptophan fluorescence increase (A (■)), Soret band shift (B), 695-nm absorbance decrease (C), and peroxidase activity increase (D (○), A (◇)). In panel D, the peroxidase activity of MP-8 as a function of [Gdn·HCl] is also shown (×). The protein unfolding data were fit to the two-state model of unfolding (*18*). See Experimental Procedures for details. [Cytc] was 3.2 μM in the fluorescence and Soret measurements and 60 μM in the 695-nm absorbance measurements.

Table 1: Parameters Obtained from Fitting Horse Heart Cytc Unfolding Curves Measured by Different Methods[a]

method	ΔG_{unf} (kcal/mol)	m (kcal/mol·M)	a^b	b^b	c^b	d^b
fluorescence	7.6 ± 0.9	2.9 ± 0.3	0.4 ± 0.6	0.4 ± 0.6	30.3 ± 1.5	5.8 ± 0.3
Soret position	8.6 ± 0.9	3.6 ± 0.4	409.3 ± 0.1	−0.59 ± 0.1	398.5 ± 0.2	0.3 ± 0.03
695-nm band	6.6 ± 0.6	2.7 ± 0.3	49.4 ± 0.4	−2.6 ± 0.8	6.5 ± 1.0	−0.14 ± 0.2
peroxidase activity	2.4 ± 0.1	1.0 ± 0.1	na[c]	na[c]	8.1 ± 0.7	0.5 ± 0.1

[a] 298 K, pH 4.5. [b] *a* and *c* correspond to the values of native and fully unfolded protein at zero denaturant concentration, respectively, and *b* and *d* to their respective dependence on [Gdn·HCl], as discussed in Experimental Procedures. [c] Not applicable, as the native state is assumed to have zero peroxidase activity.

line with earlier findings (*17*). As observed, the short peptide attached to the heme in MP-8 is not expected to experience a cooperative transition by the addition of Gdn·HCl. The accelerating effect of Gdn·HCl on the peroxidase activity of MP-8 may be related to a lowering of the pK_a of H_2O_2. This is relevant, as it is the peroxide anion that is the reactive species (*5*). This effect is small (∼9 times) compared to the effect that Gdn·HCl has on the activity of horse heart cytc (∼375 times), supporting the notion that it is unfolding that causes the large increase in activity with horse heart cytc.

Comparison of Equilibrium Unfolding of c-Type Cytochromes The dependence of the peroxidase activity on [Gdn·HCl] was compared for several Class I cytochromes *c*. In all cases, the cytochromes display an increase in peroxidase activity upon unfolding (Figure 3). When fully unfolded, at pH 4.5 the peroxidase activity is similar for all cytochromes and in the same order of magnitude as that displayed by MP-8, despite their different size and composition. The unfolded protein matrix clearly neither inhibits nor enhances the peroxidase activity of *c*-type hemes. The curves in Figure 3 were fitted according to the two-state model of unfolding, and the resulting parameters are listed in Table 2. All proteins behave similarly in that their stability as measured by peroxidase activity increase is lower than that measured by alternative (spectroscopic) methods (Table 2). Interestingly, the thermodynamic parameters for the unfolding as determined by the peroxidase activity assays are significantly lower for the mitochondrial cytochromes than for the bacterial cytochromes (Table 2). This may be related to the well-known alkaline transitions of these proteins, which for yeast and horse heart cytc take place at lower pH (pK_a ∼9 (*32*)) than that for *P. versutus* cytc-550 (pK_a ∼11.2 (*33*)) and that for *Ps. aeruginosa* cytc-551 (pK_a ∼11 (*32*)).

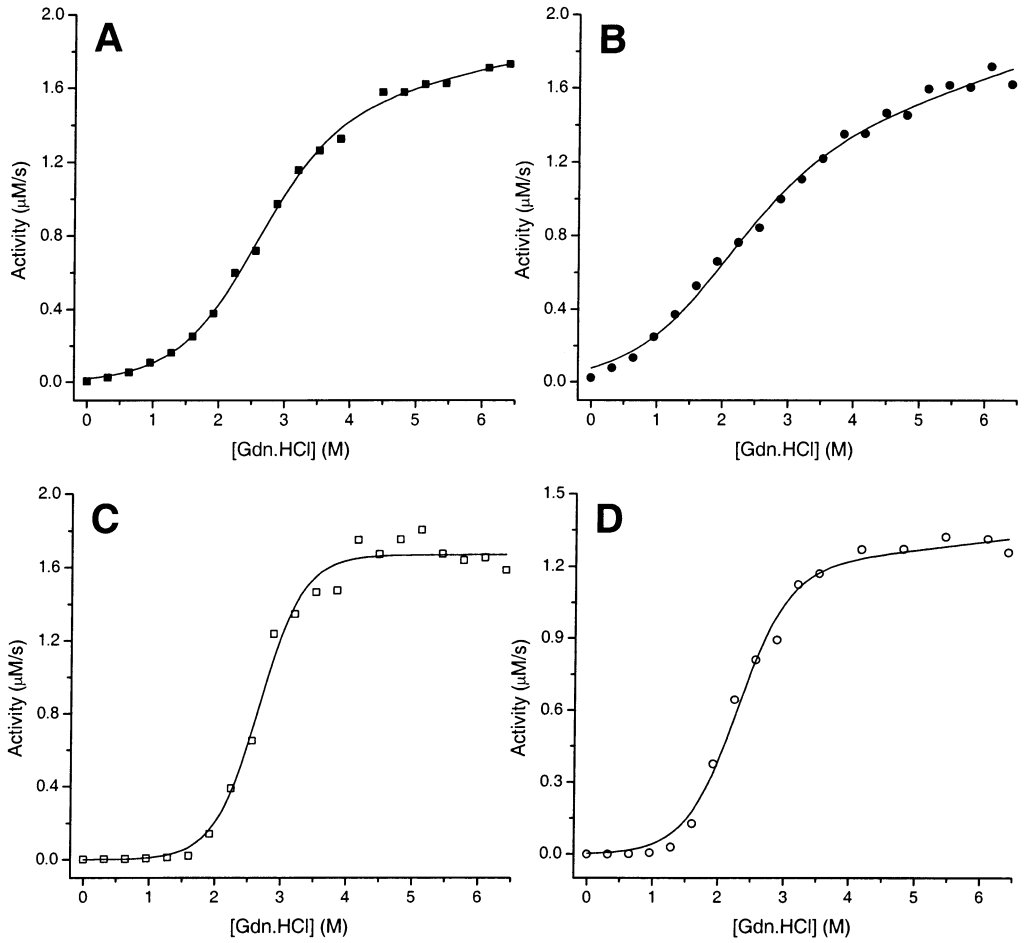

FIGURE 3: Equilibrium unfolding at pH 4.5 (298 K), as monitored by peroxidase activity increase, of horse heart cytc (A), yeast iso-1-cytc (B), *P. versutus* cytc-550 (C), and *Ps. aeruginosa* cytc-551 (D). The data were fit to the two-state model of unfolding (*18*). See Experimental Procedures for details on experiments and curve fitting.

Table 2: Thermodynamic Parameters for the Unfolding of Various c-Type Cytochromes, as Measured by Peroxidase Activity Increase at pH 4.5 and 298 K and Compared to Literature Values of Equilibrium Studies Using Alternative Methods

	peroxidase activity increase		literature value	
protein	ΔG_{unf} (kcal/mol)	*m* (kcal/mol·M)	ΔG_{unf} (kcal/mol)	*m* (kcal/mol·M)
horse heart cytc	2.4 ± 0.1	1.0 ± 0.1	10.1[a]	3.9[a]
yeast iso-1 cytc C102T	1.5 ± 0.1	0.8 ± 0.1	4.1[b]	4.3[b]
P. versutus cytc-550	4.7 ± 0.6	1.8 ± 0.2	n.a.[c]	n.a.[c]
Ps. aeruginosa cytc-551	3.4 ± 0.4	1.5 ± 0.2	6.6[d]	3.1[d]

[a] Fluorescence, 283 K, pH 5.0 (*52*). [b] CD spectroscopy, 298 K, pH 4.5 (*40*). This value is for iso-1-cytc C102S, which is slightly more stable than the C102T variant (*39*, *59*). [c] Not available. [d] Fluorescence measurements, 283 K, pH 4.7 (*53*).

Caution is required in those cases where the post-transitional baseline is very steep. This is illustrated by yeast iso-1-cytc, where the transitional midpoint measured by peroxidase activity is 1.88 M Gdn·HCl, compared to 0.95 M Gdn·HCl by CD spectroscopy (*40*), seemingly inconsistent with detection of an unfolding intermediate by peroxidase activity. The relative steepness of the post-transitional baseline, which approaches the maximal slope of the cooperative transition (Figure 3B), complicates the fitting procedure and may artificially shift the transitional midpoint. The posttransitional increase in peroxidase activity may be related to an increase in protein volume, which in turn lowers the effective concentration of protein-based heme-ligands that inhibit the activity (vide infra).

Effect of the Axial Ligand on the Unfolding-Induced Peroxidase Activity of P. versutus Cytochrome c-550. Current understanding is that the first step in cytochrome unfolding involves local unfolding of the region containing the sixth ligand and its subsequent release from the heme-iron (*9*, *22*). Replacement of the fairly weak Met ligand (*26*, *34*, *35*) by a stronger ligand may influence the unfolding transition responsible for the peroxidase activity. For this reason, the unfolding of wt and M100K cytc-550 was compared. In M100K cytc-550, the heme-iron is coordinated by a Lys ϵ-amino (*11*), which is a stronger ligand than the Met thioether sulfur (*26*).

In Figure 4A, the peroxidase activity as a function of unfolding at pH 8.0 is shown for wt and M100K cytc-550. The unfolding curves are clearly similar and both can be fitted to the two-state model of unfolding yielding similar values of ΔG and *m* (not shown). However, the total activity increase upon unfolding, when related to the activity

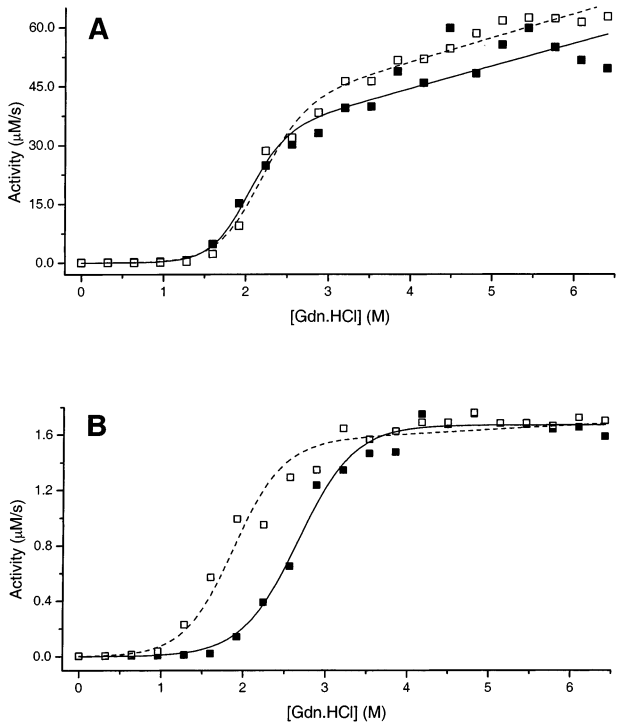

FIGURE 4: Equilibrium unfolding (298 K) monitored by peroxidase activity increase, at pH 8.0 (A) and pH 4.5 (B) of wt (■) and M100K (□) cytc-550 from *P. versutus*. Wt cytc-550 (solid line) and M100K cytc-550 (dashed line) were fitted to the two-state model of unfolding (*18*).

observed in the absence of denaturant, differs significantly between both proteins: ~1800 times for wt cytc-550 versus ~42000 times for M100K cytc-550. This signifies that the unfolding transitions are similar for both proteins, but that the peroxidase activity of M100K cytc-550 is much more inhibited by the axial ligand than wt cytc-550 at zero [Gdn·HCl] (27 and 1.5 nM s^{-1} for wt and M100K cytc550 respectively, at 0 M Gdn·HCl, and as in Figure 4A). This confirms that Lys is a stronger ligand than Met for ferric heme-iron. The fact that both proteins display similar unfolding curves may indicate that the unfolding transition is not under control of the axial ligand alone, but involves a more extensive region under these conditions.

In a second experiment, both proteins were unfolded at pH 4.5 (Figure 4B). Wt cytc-550 displays increased stability and the total increase in activity is somewhat higher than for M100K cytc-550 (~1000 vs ~625 times). This may be explained by invoking a slightly weaker axial ligation at pH 4.5 for M100K than wt cytc-550. This is in line with earlier findings (*11*) demonstrating a slightly increased pK_a of ligand exchange [the so-called state II−III transition (*20*, *32*)], indicative of a somewhat higher acid-sensitivity for the M100K mutant. At pH 4.5 this apparently translates into an altered unfolding midpoint, unlike observed at pH 8.0. Thus, with comparable inhibitory strength of the axial ligand (at pH 4.5) a difference in stability is detected, while this is not the case under conditions where the Lys of M100K cytc-550 is a stronger ligand (pH 8.0). This suggests that the region around the axial ligand is less stable in M100K than in wt cytc-550. Moreover, it verifies that local unfolding of this region is not exclusively under control of the axial ligand.

pH Dependence of Equilibrium Unfolding of Horse Heart Cytochrome c. Figure 5 shows the peroxidase activity of

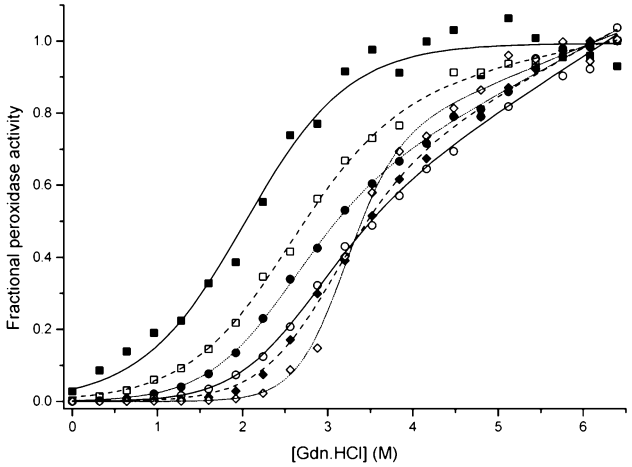

FIGURE 5: Equilibrium unfolding (298 K) of horse heart cytc, as monitored by peroxidase activity increase, at pH 4.0 (−■−), pH 4.5 (−□−), pH 5.0 (·●·), pH 6.0 (−○−), pH 7.0 (−◆−), and pH 8.0 (·◇·). The lines through the data points represent best fits to the two-state model of unfolding (*18*). See Experimental Procedures for details on experiments and curve fitting.

Table 3: pH Dependence of the Thermodynamic Parameters for the Unfolding of horse heart cytc, as Measured by Peroxidase Activity Increase (298 K)

pH	ΔG_{unf} (kcal/mol)	*m* value (kcal/mol·M)	total increase in activity (times)
4.0	2.0 ± 0.2	1.0 ± 0.1	36
4.5	2.4 ± 0.1	1.0 ± 0.1	266
5.0	2.7 ± 0.2	1.1 ± 0.1	378
6.0	3.0 ± 0.4	1.2 ± 0.2	513
7.0	3.8 ± 0.3	1.3 ± 0.1	1062
8.0	6.3 ± 0.9	2.0 ± 0.3	3341

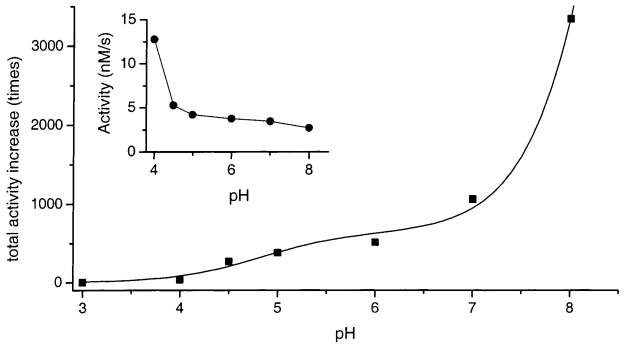

FIGURE 6: Total increase in peroxidase activity of horse heart cytc by Gdn·HCl-induced unfolding, as a function of pH (298 K). The increase was calculated from the two-state fits to the data in Figure 5, taking the post-transitional baseline into account. Inset: the pH dependence of the peroxidase activity of horse heart cytc at 0 M Gdn·HCl.

horse heart cytc as a function of Gdn·HCl at several pH values. With increasing pH, the ΔG of the transition as well as the *m*-value increases (Table 3). The total activity increase upon unfolding also becomes larger with increased pH, as shown in Figure 6. Two protonation events seem to affect the total increase in peroxidase activity, one above pH 8, the other below pH 5. It is tempting to reconcile these events with the well-known acid- and alkaline transitions of horse heart cytc. The acid transition (pK_a ~ 2.5) involves unfolding and loss of the axial ligand and the alkaline transition (pK_a ~ 9) is the exchange of Met for Lys (*20*). The acid form is thus expected to have a relatively high peroxidase activity,

and to show little activity increase when Gdn·HCl is added (in fact no increase is observed at pH 3.0, vide infra). The alkaline transition on the other hand, involves the introduction of a strong ligand, and thus unfolding will induce a larger activity increase. Thus, these unfolding curves reflect the strength of the axial ligation.

The Effect of Gdn·HCl on the Peroxidase Activity at pH 3.0. Figure 7 shows the peroxidase activity at pH 3.0 as a function of [Gdn·HCl] for the cytochromes from horse heart, yeast and *P. versutus*. An activating effect of denaturant is absent except for cytc-550. Apparently, the mitochondrial proteins are already (partially) unfolded at this pH in the absence of Gdn·HCl. This agrees with earlier reports showing that at pH 3.0 (and ~0.1 M ionic strength) the cytochromes from horse heart and yeast are (at least partly) acid-unfolded and high-spin (*36*−*40*). Apparently, at this pH, *P. versutus* cytc-550 is still mostly folded although only a small amount of denaturant suffices for unfolding.

Above the unfolding transition, increasing [Gdn·HCl] has a slightly inhibitory effect on the peroxidase activity. This is probably because the concomitant increase in ionic strength has the effect to make the acid-unfolded state more compact (*37*, *38*). This may decrease the accessibility of the heme-iron to hydrogen peroxide and thus reduce the activity. Note the trend for MP-8 (Figure 2D), where a molten globule state is presumably not possible. As obvious from Figure 7, the inhibitory effect is relatively small, in agreement with our previous statement that the protein matrix has very little effect on the peroxidase activity of unfolded *c*-type cytochromes.

pH Dependence of the Peroxidase Activity of Unfolded Cytochromes Figure 8 shows the pH dependence of the peroxidase activity of the fully unfolded (in 6 M Gdn·HCl) cytochromes from horse heart, yeast, *Ps. aeruginosa* and *P. versutus*, as well as MP-8. The bell-shaped curves observed in all cases are related to (*5*): (1) the heme-iron high to low spin transition, and (2) the pK_a of the peroxide substrate (which is lowered with respect to its value free in solution, by binding to the heme-iron). Assuming that the only active species are the high spin heme and the peroxide anion, the pH dependence of the fractions of these is described by eq 2 and 3, respectively. The activity is consequently described by eq 4, where *k* is the maximal (hypothetical) activity when $f(\text{HS cytc}) = f(HO_2^-) = 1$.

$$f(\text{HS cytc}) = \frac{[H^+]}{K_{a,app}^{cytc} + [H^+]} \quad (2)$$

$$f(HO_2^-) = \frac{K_{a,app}^{H_2O_2}}{K_{a,app}^{H_2O_2} + [H^+]} \quad (3)$$

$$\text{activity} = k[\text{cytc}]_{total}[H_2O_2]_{total}\, f(\text{HS cyct})\, f(HO_2^-) \quad (4)$$

The activity of each variant is identical within error at pH 4.5, i.e., well below the high to low spin change, and thus $pK_{a,app}^{H_2O_2}$ is equal for all cases. The data in Figure 8 were fitted to eq 4, whereby $pK_{a,app}^{H_2O_2}$ was fitted globally and *k* was fixed. The value of *k* leading to the best fits was obtained by manual iteration. This value corresponds to a peroxidase reaction rate of $\sim 2.5 \times 10^6\ M^{-1}s^{-1}$, which is close to values found for true heme-containing peroxidase enzymes (*7*).

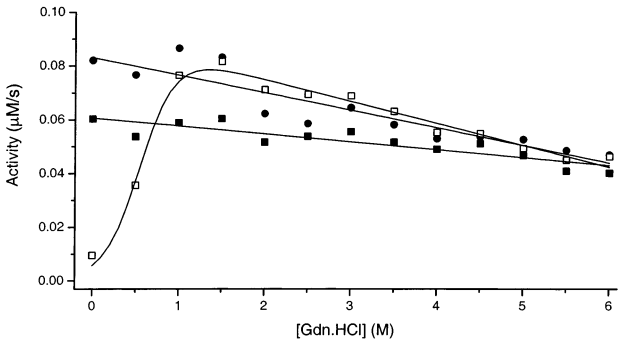

FIGURE 7: The peroxidase activity at pH 3.0 (298 K) of horse heart cytc (■), yeast iso-1-cytc (●), and *P. versutus* cytc-550 (□) as a function of [Gdn·HCl]. The lines through the data points are drawn for reasons of clarity only.

The fits (Figure 8, dashed lines) for the cytochromes from *P. versutus* and *Ps. aeruginosa* and for MP-8 are satisfactory, while the fits for the horse heart and yeast proteins are not. The model used to fit implies that the high- to low-spin transition can be described by a single deprotonation event. However, it is known that there are at least three types of ligand that induce such a transition in unfolded cytochromes. Extensive studies on yeast iso-1-cytc have shown that His residues (not including the native state His ligand, His18, horse heart numbering) are in part responsible for the spin-state change. Removal of these extra His residues by site-directed mutagenesis increased the p$K_{a,app}$ of the spin-state change by one unit (*41*). Elimination of the *N*-terminal amino group resulted in an extra 1.5 units increase (p$K_{a,app}$= 7.4) (*42*). The third ligand group is thought to be composed of ϵ-amino groups of Lys residues (*10*, *42*). The position of extra His residues on the polypeptide chain also influences the p$K_{a,app}$ of the spin-state transition in unfolded horse heart cytc and yeast iso-1-cytc (*43*−*45*). No shift in p$K_{a,app}$ is discernible between the unfolded yeast iso-1-cytc mutant containing zero additional His residues (not counting His18) and the mutant with a single additional His far away in sequence (*44*).

With this in mind, it is clear why the fits in Figure 8 (dashed lines) are so poor for the horse heart and yeast proteins. Of the five investigated molecules, only MP-8 and *Ps. aeruginosa* cytc-551 do not contain endogeneous His residues other than the native-state His ligand. *P. versutus* cytc-550 contains just one, His118, which is located so far away in sequence from the heme-binding site (*21*) that it is reasonable to expect no contribution to the spin-state change. Indeed, the activity profiles of *Ps. aeruginosa* cytc-551 and *P. versutus* cytc-550 are indistinguishable (Figure 8). Horse heart cytc contains two, His26 and His33, and yeast iso-1-cytc contains three endogeneous His residues close to the heme-binding site (His26, His33, and His39, horse heart numbering) (*20*). In accordance, the peroxidase activity of yeast iso-1-cytc is more repressed than horse heart cytc and both are lower than the activities of the *Pseudomonas* and *Paracoccus* proteins (Figure 8). The peroxidase activity of MP-8 at neutral pH is even higher, which agrees with the absence of Lys residues in this molecule (*26*).

The nature of the strong-field axial ligand and its tendency to inhibit peroxidase activity can thus be differentiated. To account for this, a second equation was devised (eq 5). For

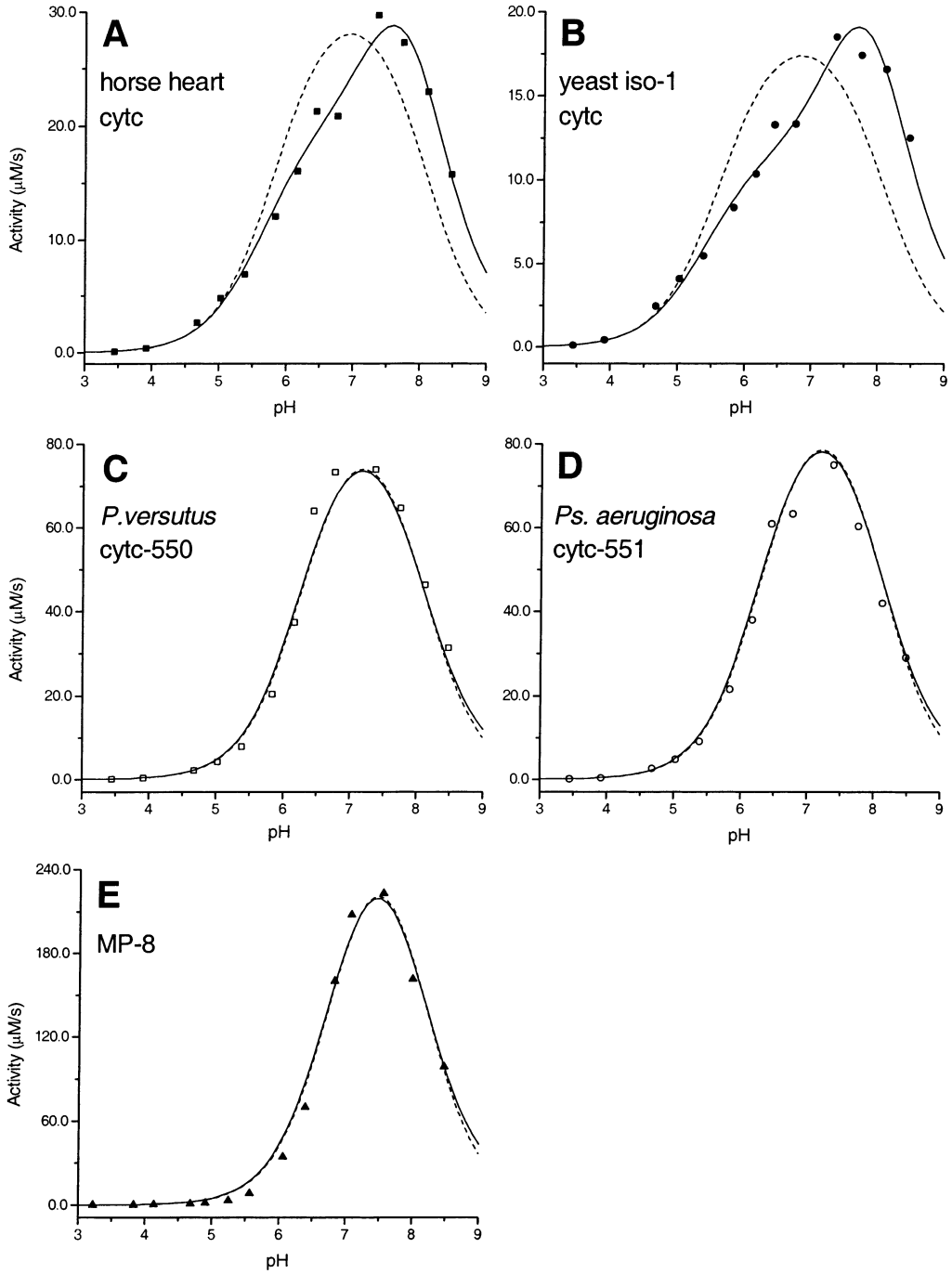

FIGURE 8: pH dependence of the peroxidase activity under denaturing conditions (6.0 M Gdn·HCl, 298 K), for horse heart cytc (A), yeast iso-1-cytc (B), *Ps. aeruginosa* cytc-551 (C), *P. versutus* cytc-550 (D), and MP-8 (E). Dashed and solid lines represent the best fits to eq 4 (using eq 2) and eq 4 (using eq 5), respectively.

simplicity, only two ligand groups are taken in account. One of these comprises His residues, and the other includes the supposed ligands of the *N*-terminal and Lys side chain amino-groups. The propensity of either ligand group is assessed in eq 5 by the ratio of effective concentrations and K_d for heme-iron binding.

$$f(\text{HS cytc}) = \left(1 + \frac{[\text{L}]_t}{K_d^{\text{L}}}\left(\frac{K_a^{\text{L}}}{K_a^{\text{L}} + [\text{H}^+]}\right) + \frac{[\text{His}]_t}{K_d^{\text{His}}}\left(\frac{K_a^{\text{His}}}{K_a^{\text{His}} + [\text{H}^+]}\right)\right)^{-1} \quad (5)$$

The data in Figure 8 were fitted to eq 4 into which eq 5 was incorporated. Parameter k (eq 4) was fixed ($\sim 2.5 \times 10^6$ $M^{-1}s^{-1}$, as above), and $pK_{a,app}^{H_2O_2}$ and the apparent pK_a's of the two ligand groups were fitted globally. The best fits are the solid lines in Figure 8, and the resulting parameters are shown in Table 4. The use of eq 5 instead of eq 2 leads to substantially better fits for the horse heart and yeast cytochromes (Figure 8A,B), while the other cases show no improvement. The value of $pK_{a,app}^{H_2O_2}$ is not changed when eq 5 is used instead of eq 2. The calculated value for pK_a^{His} is 7.1, identical to that measured in 6 M Gdn·HCl (*44*). The pK_a value for the alternative ligand is 9.3, in the range expected for amino groups (*46*).

Table 4: Parameters Obtained from Fitting the pH Dependence of the Peroxidase Activity of Unfolded Cytochromes (298 K)

protein	parameters derived from eq 4 (using eq 2)	
	$pK_{a,app}^{H_2O_2}$	$pK_{a,app}^{cytc}$ [a]
horse heart cytc	8.04 ± 0.02	5.86 ± 0.04
yeast iso-1 cytc C102T	8.04 ± 0.02	5.64 ± 0.05
P. versutus cytc-550	8.04 ± 0.02	6.33 ± 0.02
Ps. aeruginosa cytc-551	8.04 ± 0.02	6.36 ± 0.02
microperoxidase-8	8.04 ± 0.02	7.03 ± 0.03

protein	parameters derived from eq 4 (using eq 5)				
	$pK_{a,app}^{H_2O_2}$	pK_a^{His}	pK_a^{L}	$[His]_t/K_d^{His}$	$[L]_t/K_d^{L}$
horse heart cytc	8.00 ± 0.02	7.1 ± 0.2	9.3 ± 0.2	20 ± 8	1594 ± 640
yeast iso-1 cytc C102T	8.00 ± 0.02	7.1 ± 0.2	9.3 ± 0.2	41 ± 17	2082 ± 860
P. versutus cytc-550	8.00 ± 0.02	n.a.[b]	9.3 ± 0.2	0	987 ± 408
Ps. aeruginosa cytc-551	8.00 ± 0.02	n.a.[b]	9.3 ± 0.2	0	921 ± 381
microperoxidase-8	8.00 ± 0.02	n.a.[b]	9.3 ± 0.2	0	196 ± 80

[a] $pK_{a,app}^{cytc}$ is the apparent pK_a for the heme high-spin to low-spin transition. [b] Not applicable.

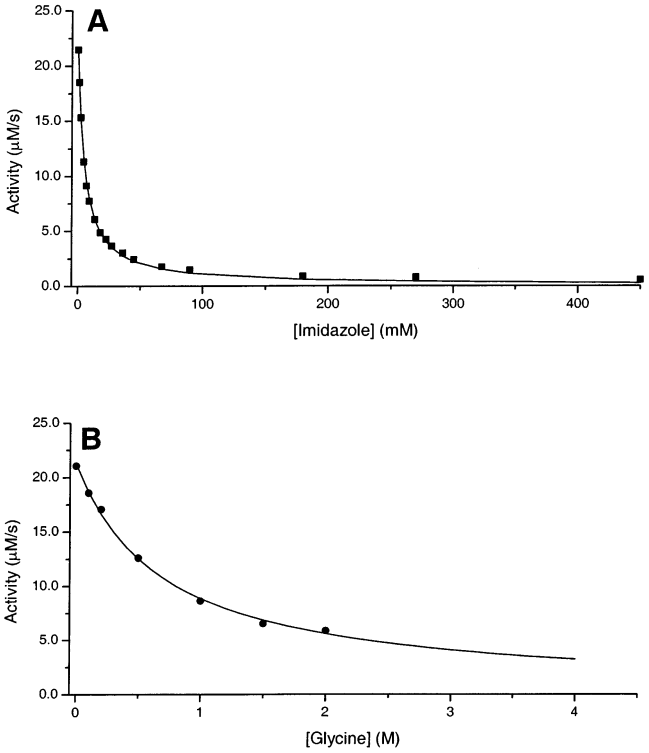

FIGURE 9: Inhibition of the peroxidase activity of unfolded (6.0 M Gdn·HCl) *P. versutus* cytc-550 at pH 5.83 (298 K) by imidazole (A) and glycine (B), respectively. In each case, the data were fitted (solid lines) to a single binding equilibrium, yielding dissociation constants of 4.9 (± 0.1) mM for imidazole and 716 (± 30) mM for glycine.

An interesting consequence of eq 5 is that it distinguishes two types of ligands, and that it provides an impression of the relative binding strength of each ligand type to the heme-iron. The calculated values of $[His]_t/K_d^{His}$ and $[L]_t/K_d^{L}$ (see Table 4), suggest that the endogeneous His are weaker ligands than the "amino group" and are displaced by the latter at alkaline pH.

The relative values of $[His]_t/K_d^{His}$ of the four cytochromes qualitatively agree with the expected values (Table 4). Only the eukaryotic cytochromes contain His residues competent for heme binding, and horse heart cytc has one His residue less than yeast iso-1-cytc. For the other ligand group, note that both horse heart cytc and *P. versutus* cytc-550 do not have *N*-termini that can coordinate the heme-iron, since they are *N*-acetylated (*19*) or pyroglutamated (*21*, *47*). This is relevant, because on average the pK_a's for N-termini are over 2 units lower compared to Lys ϵ-amino groups (*46*) and thus an N-terminus is expected to have a much stronger apparent affinity for the heme iron than a Lys N^{ϵ}. Cytc-550 contains about twice as many Lys residues as cytc-551 (15 and 7, respectively), while the values of $[L]_t/K_d$ obtained from the fits are identical within error for both proteins (Table 4). This signifies the relative importance of the N-terminus as a ligand in unfolded cytochromes, in agreement with earlier findings (*42*).

Inhibition of the Peroxidase Activity of Unfolded P. versutus Cytochrome c-550 *by Imidazole and Glycine.* To corroborate the conclusion that endogeneous His residues as well as amino-groups inhibit peroxidase activity by binding to the heme iron, the inhibitory effect of exogenous imidazole and the *N*-terminus of glycine on the peroxidase activity of unfolded *P. versutus* cytc-550 was measured. Cytc-550 was used because the endogeneous extra His does not seem to participate in inhibiting the peroxidase activity (vide supra). Figure 9A,B shows the inhibition of cytc-550 peroxidase activity at pH 5.83 by imidazole and glycine, respectively.

The inhibition by imidazole was fitted to an apparent K_d of 4.9 (±0.1) mM. Using $pK_a^{His} = 7.1$ (from Table 4) this amounts to $K_d = 250$ (±5) μM for imidazolate. This value is slightly higher than the apparent K_d's for exogenous imidazole reported for the heme-peptides MP-6 (80 μM, pH 8.5 (*48*)) and MP-11 (120 μM, pH 9.0, (*49*)), respectively. Using $K_d = 250$ μM, and the values of $[His]_t/K_d$His from Table 4, the effective concentration of His can be estimated in horse heart and yeast cytc. These are 5.0 (± 1.5) and 10.3 (± 3.8) mM, respectively. The latter value is in good agreement with an earlier estimate (*50*).

The inhibition of the peroxidase activity by glycine was fitted to an apparent K_d of 716 (± 30) mM. Assuming a value of 9.3 for its pK_a (Table 4), it emerges that the deprotonated *N*-terminus of glycine binds the heme-iron with $K_d = 243$ (± 10) μM. This value is in reasonable agreement with that established earlier for MP-11 (930 μM, pH 9.0, (*49*)).

Apparently, there is little difference in the binding affinity of imidazolate and the deprotonated *N*-terminus of glycine

to the heme iron of unfolded cytc550. This makes it likely that with increasing pH, amino-groups replace the endogeneous His ligands in unfolded, low-spin horse heart cytc and yeast iso-1-cytc, as these proteins contain an abundance of Lys residues. This is in accordance with the preceding discussion of the pH dependence of the peroxidase activity of unfolded horse heart cytc and yeast iso-1-cytc. Coordination of amino ligands to the heme-iron of *c*-type cytochromes is not unprecedented. In cytochrome *f* the sixth ligand is the N-terminal amino (*51*), and the relatively low pK_a of the alkaline transition in mitochondrial cytochromes *c* indicates that the native-state Met S^{δ} ligand can be easily replaced by a Lys N^{ϵ}.

CONCLUDING REMARKS

This study describes a novel way to look at unfolding of *c*-type cytochromes. The peroxidase activity method detects the presence of an unfolding intermediate invisible in optical and fluorescence measurements but observable in NMR studies (*9*, *10*). It corroborates that the free energy difference between this intermediate and the native state depends on the strength of the axial ligand (as described by Xu et al. (*34*)) and that acid-unfolded cytc is sensitive to increasing ionic strength (*37*). Finally, it supports the finding (*41*) that at least two types of ligands may coordinate the heme-iron under denaturing conditions.

Peroxidase activity measurements prove a useful addition to the array of techniques currently available for the study of cytc unfolding. A drawback of this technique is that only equilibrium unfolding can be studied. In addition, only the ferric form of *c*-type cytochromes is open to study, because that is the form reactive with H_2O_2. Obvious advantages are the ease of experimentation and the need for only small quantities of protein.

An important conclusion is that the peroxidase activity of *c*-type cytochromes is an extremely sensitive parameter to study intermediates in cytc unfolding. Most techniques detect mixed signals of the unfolding states energetically available under particular conditions. Generally, intermediate unfolding states are poorly populated, and they thus easily escape detection. The peroxidase activity, however, specifically relates to non-native, five-coordinate species. Peroxidase activity assays are thus most informative under conditions that favor the native state of the protein. This is where future studies will be directed.

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