Effect of the protein matrix of cytochrome c in supressing the inherent peroxidase activity of its heme group

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better insight into sequence – structure – function relationships. Cumulative substitutions of the various functional groups tested here would be one experimental approach. Crystallization of those complexes that largely deviate from the theoretical modeling could be another goal.

Experimental Section

Chemical synthesis: Treatment of thymidine with *p*-tolylsulfonyl chloride in pyridine at 0 °C yielded the 5′-sulfonate derivative **1** (72%). Displacement of the 5′-sulfonyl group with sodium azide in DMF at 80 °C gave the 5′-azido derivative **2** (79%). Hydrogenation of **2** in ethanol in the presence of palladium on charcoal resulted in an 83% yield of 5′-amino-2′,5′-dideoxythymidine (5′NH_2-dT, **3**). Acetylation of **3** with acetic anhydride followed by mild saponification yielded the *N*-acetylated derivative **4** (78%). Compounds **1**, **2**, and **4** were purified by silica gel chromatography. Compound **3** was purified by reversed-phase high pressure liquid chromatography. All nucleoside analogues were characterized by NMR spectroscopy.

TMPK assays: The reaction medium (0.5 mL final volume) contained 50 mM Tris(hydroxymethyl)aminomethane-HCl pH 7.4, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM NADH, 1 mM phosphoenol pyruvate, and 2 units each of lactate dehydrogenase, pyruvate kinase, and nucleoside diphosphate kinase. Activity was determined by using the coupled spectrophotometric assay at 334 nm (0.5 mL final volume) in an Eppendorf ECOM 6122 photometer.[10] One unit of enzyme activity corresponds to 1 μmole of the product formed in 1 minute at 30 °C and pH 7.4. The concentration of ATP was kept constant at 0.5 mM. The concentrations of dT analogues and dTMP were varied between 0.05 and 0.8 mM.

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Effect of the Protein Matrix of Cytochrome *c* in Suppressing the Inherent Peroxidase Activity of Its Heme Prosthetic Group

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KEYWORDS:

cytochrome *c* · heme proteins · oxidative stress · protein folding · radicals

Free heme is a potent peroxidase; it readily reacts with H_2O_2 and is then capable of a wide variety of oxidation reactions.[1] The heme group of cytochrome *c* (cytc) can be expected to be an equally competent producer of free radicals, but its intrinsic peroxidase activity is suppressed by the protein matrix.[2] This is of vital importance to the living cell because free radicals derived from oxygen play a key role in aging and its pathophysiology.[3] Herein, we quantify the suppression of the inherent peroxidase activity of heme by the protein matrix of cytc. We show that the peroxidase activity of unfolded cytc is similar to that of microperoxidase-8 (MP-8).[4]

The protein used here is the well-studied cytochrome *c*-550 (cytc550) from *Paracoccus versutus*.[5] Its peroxidase activity has been characterized.[2a] It is a Class I cytc, closely related both to the archetypal mitochondrial cytc proteins, and to numerous other, bacterial *c*-type cytochromes.[6]

Addition of the common denaturing agent guanidinium hydrochloride (Gdn·HCl) to ferricytc550 causes the protein to unfold, as was observed by using the following indicators (Figure 1).[7] In Figure 1A, the tryptophan fluorescence is plotted as a function of Gdn·HCl concentration. Unfolding causes the structure of cytc550 to expand and leads to an increased average distance between its tryptophan residues and the covalently bound heme group. This results in a less efficient energy transfer from the tryptophan residues to the heme moiety, and consequently the tryptophan fluorescence increases upon unfolding.[8] Secondly, unfolding leads to changes in the optical spectrum. The dominant effect is a transition of the heme iron center from low spin to high spin, caused by the loss of the native methionine ligand (Figure 1B).[9, 10] Finally, Figure 1C demonstrates that an increase in Gdn·HCl concentration causes the peroxidase activity of cytc550 to rise dramatically (about 1200-fold).[7] No such activity is seen when any of the components (Gdn·HCl, cytc550, H_2O_2, or the reducing substrate, guaiacol (gc)) are left out of the reaction mixture.[7] The activity

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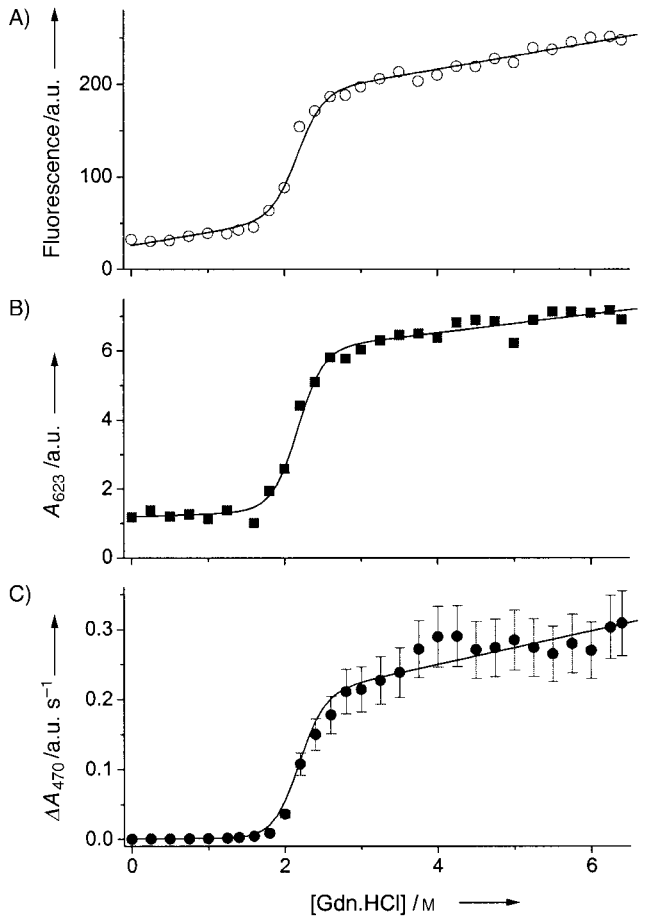

Figure 1. *Equilibrium unfolding of ferricytc550 by Gdn·HCl. A) Fluorescence (excitation: 295 nm, emission: 360 nm). B) Optical absorbance. The intensity of the band at 623 nm is typical of high-spin heme groups.[9] C) Peroxidase activity, that is, spectrophotometrical measurement of the absorbance of oxidized guaiacol (tetraguaiacol) at 470 nm after addition of H_2O_2. All experiments were performed in 100 mM sodium phosphate (pH 5.0, 25 °C). [cytc550] was 2 μM in the fluorescence and UV/Vis measurements, and 1 μM in the peroxidase assay (with 10 mM H_2O_2 and 10 mM guaiacol). Data were fitted (solid lines) assuming a two-state model of unfolding with linear baselines.[11]*

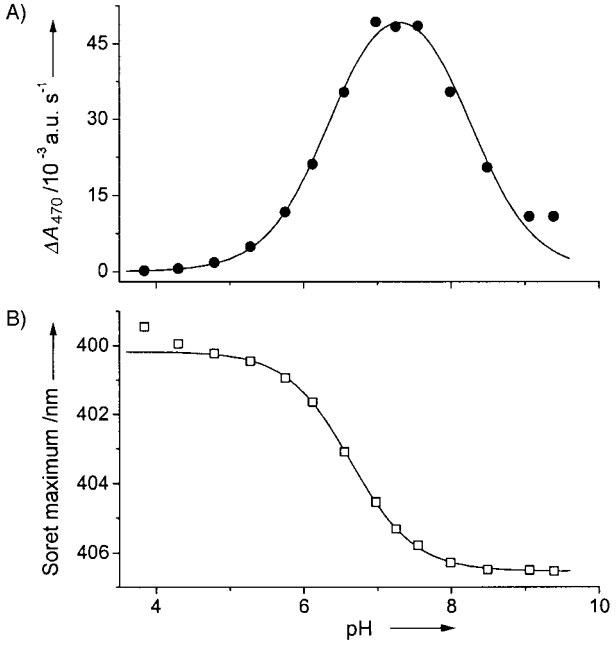

Figure 2. *pH dependence of unfolded cytc550. A) Peroxidase activity. Concentrations of cytc550, H_2O_2, and guaiacol were 1 μM, 0.1 mM, and 10 mM, respectively, in 100 mM sodium phosphate and 6 M Gdn·HCl (25 °C). The data (●) were fitted assuming that only high-spin cytc550 and HO_2^- are active (——). The fit yielded pK_a values of 6.4(±0.1) and 8.3(±0.1) for the spin-state transition and H_2O_2 deprotonation,[12] respectively. B) Position of the main visible absorption band (Soret) of unfolded cytc550. The data (□) were fitted to a single pK_a value of 6.6(±0.2) (——).*

increase seen in Figure 1C correlates well with the unfolding monitored by fluorescence and UV/Vis spectroscopy (Figure 1A, B).[11]

The peroxidase activity of fully unfolded cytc550 depends strongly on the pH value (Figure 2A). The shape of the curve is ascribed to the involvement of two acid–base transitions. One of these transitions is the change from high to low spin undergone by unfolded cytc550 as the pH value is increased (Figure 2B).[9] This transition presumably involves occupation of the sixth coordination position of the heme iron center by a strong ligand,[9] which blocks the incoming peroxide from that site. The second acid–base transition is thought to involve deprotonation of H_2O_2.[12] Like other small inorganic ligands, the HO_2^- anion is expected to bind much more strongly to the heme-iron than H_2O_2 (compare with CN^- versus HCN).[13] This is reflected in the mode of action of natural peroxidases, in which the distal histidine plays a very important role as a base catalyst and deprotonates the incoming H_2O_2.[14]

To assess the kinetic mechanism of the peroxidase activity of unfolded cytc550, the dependence of the reaction rate on gc and H_2O_2 concentrations was determined (Figure 3). The data fit satisfactorily (solid lines) to a ping-pong mechanism.[15] The H_2O_2 concentration at which the reaction rate is half its maximum, $K_M^{H_2O_2}$, is much lower for unfolded cytc550 at pH 5.0 (236 ± 14 mM) than for native cytc550 (≫1 M, data not shown). These values

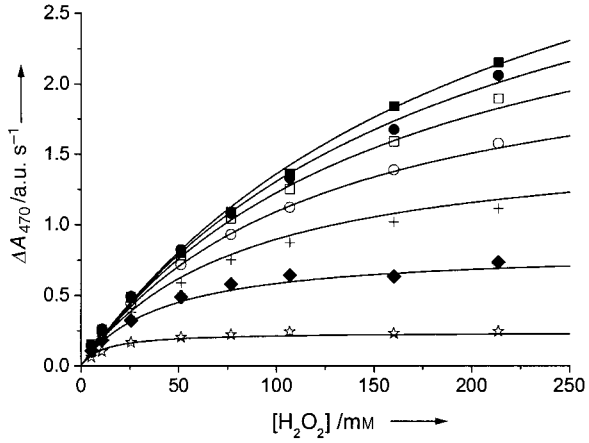

Figure 3. *Peroxidase activity as a function of H_2O_2 and guaiacol. Conditions: 1 μM cytc550, 100 mM sodium phosphate (pH 5.0), and 6 M Gdn·HCl (25 °C). H_2O_2 dependence was probed at 0.25 (*), 1.0 (◆), 2.5 (+), 5.0 (○), 10.0 (□), 20.0 (●), and 50.0 (■) mM guaiacol. All datapoints were fitted together to a ping-pong mechanism.[15]*

indicate that unfolding leads to an active site that is much more accessible to the substrate.

It is interesting to compare the peroxidase activities of unfolded cytc550, MP-8,[4, 7b] and native cytc550[2a] with each other. Below 1 mM H_2O_2 the activity of all three species is described by a bimolecular rate equation.[16] It emerges that the bimolecular rate constant k_{obs}[16] of unfolded cytc550 ($3 \times 10^3\,M^{-1}s^{-1}$; pH 5.0, 10 mM gc, 6 M Gdn·HCl) is similar to that of MP-8 ($1.3 \times 10^3\,M^{-1}s^{-1}$; pH 5.5, 3–4.5 mM gc, 0.5 M Gdn·HCl)[7b] but much larger than that of native cytc550 ($2.6\,M^{-1}s^{-1}$; pH 5.0, 25 mM gc, data not shown). Unfolding the protein surrounding the heme group of cytc550 thus has the same effect as clipping the protein away (as in MP-8). In both cases, the peroxide substrate has improved access to the heme iron center in the absence of both a rigid protein matrix and a strong sixth ligand.

Native cytc550 can catalyze H_2O_2-driven oxidations, but with a very low rate of reaction.[2a] Clearly, the protein matrix provides excellent suppression (>1000-fold) of the inherent peroxidase activity of the heme group. The key factor is probably that the correctly folded protein provides a strong sixth ligand, which blocks the heme iron center from the incoming peroxide molecule. This conclusion was apparent from the pH dependence of the peroxidase activity under denaturing conditions: low-spin unfolded cytc550, the principal species at high pH values, has a negligible activity compared to high-spin unfolded cytc550. Thus, even when it is unfolded, cytc550 has no peroxidase activity when a strong sixth ligand is present (as in the low-spin case).

The Gibbs free energy for unfolding (ΔG_{unf}) is 6.6 kcal mol^{-1} for cytc550 when the two-state unfolding model is applied (Figure 1). Under native conditions, this corresponds to one unfolded species for every 70000 protein molecules. In 6 M Gdn·HCl, however, the activity is approximately 1200-fold higher than normal, and not 70000-fold. Therefore, under native conditions partially unfolded, high-spin species are present which dominate the peroxidase activity. In agreement with this conclusion, the region around the native methionine ligand is known to have a lower ΔG_{unf} than the global ΔG_{unf} for Class I cytc proteins such as cytc550.[17]

Any environmental stimulus which leads to the formation of (partially) unfolded species is likely to induce peroxidase activity in cytc550 and hence may stimulate free-radical formation in the living cell. It is the robustness of the native protein structure that prevents peroxidase activity in cytc550.

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[7] a) All chemicals were of the highest grade commercially available. A stock solution of 8 M Gdn·HCl (Biochemika grade, Fluka) was prepared and filtered (0.45 μm HV Durapore, Millipore) before use. Cytc550 was expressed and purified, and peroxidase activity was assayed as described in ref. [2a]. The assay utilized H_2O_2 and *o*-methoxyphenol (guaiacol (gc), Sigma) as oxidizing and reducing substrates, respectively, to give a fourfold oxidized product ($\varepsilon_{470} = 26.6\,mM^{-1}cm^{-1}$);[7b] b) D. A. Baldwin, H. M. Marques, J. M. Pratt, *J. Inorg. Biochem.* **1987**, *30*, 203.
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[11] a) Data were fitted to a two-state unfolding model assuming linear baselines for native and unfolded protein.[11b] All data sets were fitted together, to yield values of 6.6(±0.8) kcal mol^{-1} and 3.1(±0.3) kcal mol^{-1} M^{-1} for ΔG_{unf} and *m* respectively; b) M. M. Santoro, D. W. Bolen, *Biochemistry* **1992**, *31*, 4901.
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[15] a) Equation (1) was used for the fit in Figure 3.[15b]

$$\text{rate} = (V_{max} \times [H_2O_2] \times [gc])/((K_M^{gc} \times [H_2O_2]) + (K_M^{H_2O_2} \times [gc]) + ([H_2O_2] \times [gc])) \quad (1)$$

The values obtained were: $k_{cat} = V_{max}/[\text{cytc550}] = 707(\pm 26)\ s^{-1}$, $K_M^{H_2O_2} = 236(\pm 14)$ mM, and $K_M^{gc} = 4.7 \pm 0.3$ mM; b) A. Cornish-Bowden, *Fundamentals of Enzyme Kinetics*, revised ed., Portland, London, **1995**, p. 146.
[16] When $[H_2O_2] \ll K_M^{H_2O_2}$ and [gc] is sufficiently high, the rate equation[15a] reduces to Equations 2 and 3.

$$\text{rate} = k_{obs} \times [\text{heme}] \times [H_2O_2] \quad (2)$$

$$k_{obs} = k_{cat}/K_M^{H_2O_2} \quad (3)$$

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