Proton Linkage in Formation of the Cytochrome *c*–Cytochrome *c* Peroxidase Complex: Electrostatic Properties of the High- and Low-Affinity Cytochrome Binding Sites on the Peroxidase†

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Received May 12, 1994; Revised Manuscript Received July 25, 1994®

ABSTRACT: The electrostatic character of cytochrome *c*-cytochrome *c* peroxidase complex formation has been studied by potentiometric titration between pH 5.5 and 7.75. Potentiometric data obtained at ionic strength ≥100 mM were adequately analyzed in terms of 1:1 complex formation while the simplest model capable of fitting similar data obtained at lower ionic strength involves the assumption of two inequivalent binding sites for the cytochrome on the peroxidase. The stability of cytochrome *c* binding at the high-affinity site is ca. three orders of magnitude greater than that observed for the low-affinity site and is optimal between pH 6.75 and 7. The electrostatic properties of the two binding sites are distinctly different because, at most values of pH, binding of cytochrome *c* to the high-affinity site results in proton release while binding of the cytochrome to the low-affinity site results in proton uptake. Furthermore, binding of the cytochrome to the low-affinity site appears to be least stable in the pH range where binding to the high-affinity site is optimal. Interestingly, the binding parameters derived from these measurements were independent of temperature, consistent with a substantial entropic contribution to complex stability. Ferricytochrome *c* binds to the peroxidase with a slightly greater affinity than does ferrocytochrome *c*, and no evidence for specific anion effects on complex stability was observed. At low ionic strength (≤50 mM) and high pH (7.75), the interaction of the two proteins is more complex and cannot be adequately analyzed in terms of the two-site model.

Since the pioneering work by Mochan and Nichols (Mochan & Nichols, 1971; Mochan, 1970) and Yonetani and co-workers (Yonetani & Leonard, 1973; Gupta & Yonetani, 1973), the complex formed by cytochrome *c* and cytochrome *c* peroxidase has served as a paradigm for similar complexes formed by other electron transfer proteins. Particular attention was focused on this complex by development of the hypothetical structure for this complex proposed by Poulos and Kraut (1980) on the basis of the three-dimensional structures of the two component proteins. While the recent determination of three-dimensional structures for crystallized forms of the cytochrome *c*–cytochrome *c* peroxidase complex (Pelletier & Kraut, 1992) constitutes a major advance in our understanding of this complex, a variety of functional studies provide compelling evidence for the existence of a second cytochrome binding site on the peroxidase (Kang et al., 1977; Kornblatt & English, 1987; Stemp & Hoffman, 1993; Zhou & Hoffman, 1993, 1994) that has not been detected crystallographically.

Despite this diverse range of physical studies concerning the properties of the interaction of these two proteins, little or no attention has been directed at assessing the dependence of cytochrome *c*-cytochrome *c* peroxidase complex formation on pH or temperature. As assembly of this complex involves the interaction of two highly and oppositely charged protein surfaces, it is reasonable to expect that the stability and mechanism of complex formation should be proton-linked in a fashion similar to that which we have recently reported for formation of the cytochrome *c*–cytochrome b_5 complex (Mauk et al., 1991). The thermodynamic parameters for formation of this complex are also of considerable interest because the significant displacement of solvent from the interacting surfaces of the two proteins can be expected to provide a significant entropic component to the formation of the complex as has previously been observed for the cytochrome *c*–cytochrome b_5 complex (Mauk et al., 1984). To address these issues and, in particular, to define the electrostatic properties of cytochrome *c*–cytochrome *c* peroxidase complex formation, we now report a potentiometric study of this interaction.

EXPERIMENTAL PROCEDURES

Recombinant yeast iso-1-cytochrome *c* was prepared from transformed yeast as described previously (Rafferty et al., 1990). This form of the protein possessed a Thr residue in place of the Cys residue present at position 102 in the wild-type protein to reduce autoreduction of the ferricytochrome and eliminate dimerization through intermolecular disulfide bond formation (Cutler et al., 1987). Recombinant cytochrome *c* peroxidase was expressed in *Escherichia coli* and purified as described previously (Fishel et al., 1987; Ferrer et al., 1994). The final product obtained after 3–5 crystallizations exhibited an electronic spectrum with absorption maxima and absorption ratios identical to those defined by Vitello et al. (1990) except that the band reported to occur at 645–647 nm occurred at 642 nm. The concentrations of cytochrome *c* (Margoliash & Frohwirt, 1959) and cytochrome *c* peroxidase (Vitello et al., 1990) solutions were determined from reported extinction coefficients.

The binding of cytochrome *c* to cytochrome *c* peroxidase was studied by the potentiometric method initially developed by Laskowski and co-workers (Laskowski & Finkenstadt, 1972; Lebowitz & Laskowski, 1962) as we have described

† This work was supported by NIH Grant GM33804.

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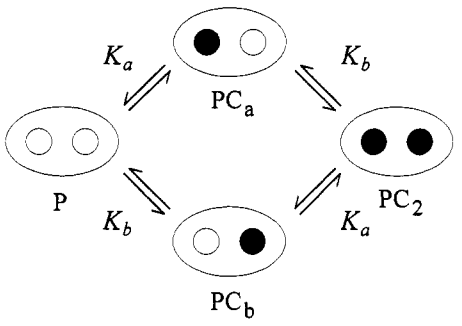
® Abstract published in *Advance ACS Abstracts,* September 15, 1994.

0006-2960/94/0433-12609$04.50/0

Scheme 1



previously for analysis of cytochrome *c*–cytochrome b_5 complex formation (Mauk et al., 1991). The resulting potentiometric data were analyzed by fitting to either a one-site or a two-site model with the program SCIENTIST (MicroMath, Salt Lake City, UT). Analysis with the one-site model has been described previously (Mauk et al., 1991). Analysis with the two-site model (Klotz, 1986) to derive so-called site binding constants is described below.

The binding of cytochrome *c* (C) to two nonequivalent, noninteracting binding sites, *a* and *b*, present on cytochome *c* peroxidase (P) can be described by the equilibria depicted in Scheme 1. The following relationships can be written to describe these equilibria:

$$K_a = \frac{PC_a}{C \times P} = \frac{PC_2}{C \times PC_b} \quad (1)$$

$$K_b = \frac{PC_b}{C \times P} = \frac{PC_2}{C \times PC_a} \quad (2)$$

$$P_t = P + PC_a + PC_b + PC_2 \quad (3)$$

$$C_t = C + PC_a + PC_b + 2PC_2 \quad (4)$$

At equilibrium, P and C represent the concentrations of the ligand-free proteins, PC_a and PC_b represent the concentrations of the possible 1:1 complexes, and PC_2 represents the concentration of the 2:1 complex. P_t and C_t are the total concentrations of the two proteins. The mole fraction of exchanged protons (H_e^+) in the overall binding process can be expressed as the sum of the individual contributions from each of the complexes formed as follows:

$$H_e^+ = q_a\frac{PC_a}{P_t} + q_b\frac{PC_b}{P_t} + (q_a + q_b)\frac{PC_2}{P_t} \quad (5)$$

where q_a and q_b represent the limiting values of the mole fraction of exchange protons for sites *a* and *b*. In a titration experiment where C is added to P, the total concentration of C (C_t) can be expressed at any time in terms of the total concentration of P (P_t) and the mole fraction of titrant to titrand (R) as $C_t = P_tR$.

Although an analytical solution of eqs 1–5 for K_a, K_b, q_a, and q_b can be derived in terms of the independent variables P_t and R, the resulting relationship is sufficiently complex that we have, instead, rearranged eqs 1–5 as follows and retained PC_2 as an implicit variable:

$$PC_b = \frac{K_b}{K_a}PC_a \quad (6)$$

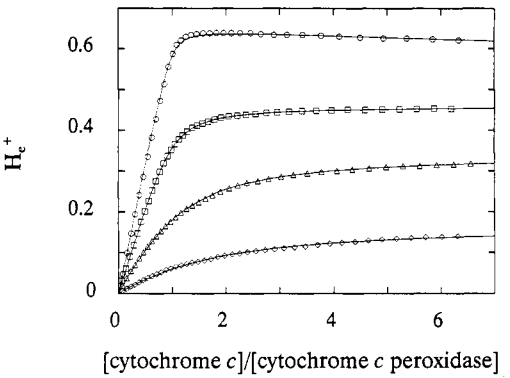


FIGURE 1: Effect of ionic strength on proton release during the titration of cytochrome *c* peroxidase with ferricytochrome *c* (pH 5.99, 25 °C, KNO_3): (○) μ = 50 mM, $P_{initial}$ = 34.3 μM; (□) μ = 100 mM, $P_{initial}$ = 77.8 μM; (△) μ = 150 mM, $P_{initial}$ = 60.2 μM; (◇) μ = 200 mM, $P_{initial}$ = 70.0 μM. H_e^+ represents the number of protons released to the medium per mole of titrand protein. The curve drawn with the 50 mM ionic strength data is the unweighted least-squares fit to the model describing two noninteracting cytochrome *c* binding sites per cytochrome *c* peroxidase molecule. The curves drawn for the higher ionic strengths are least-squares fits to the model for a single cytochrome *c* binding site.

$$PC_a = \frac{1}{2\left(1 + \frac{K_b}{K_a}\right)^2}\left[\left[\frac{1}{K_a} - \left(1 + \frac{K_b}{K_a}\right)(3PC_2 - P_tR - P_t)\right] - \left\{\left[\frac{1}{K_a} - \left(1 + \frac{K_b}{K_a}\right)(3PC_2 - P_tR - P_t)\right]^2 - 4\left(1 + \frac{K_b}{K_a}\right)^2[PC_2(2PC_2 - P_tR - 2P_t) + P_t^2R]\right\}^{1/2}\right] \quad (7)$$

$$PC_2 = \frac{K_bPC_a}{1 + 2K_bPC_a}\left[P_tR - PC_a\left(1 + \frac{K_b}{K_a}\right)\right] \quad (8)$$

RESULTS

The effect of ionic strength on the net change in proton binding that occurs upon addition of cytochrome *c* to cytochrome *c* peroxidase at pH 5.99 (25 °C) is shown in Figure 1. Under the conditions used in these measurements, proton release is observed in each case, though the number of binding sites that must be invoked to fit the data adequately is a function of ionic strength. Data collected at $\mu \geq 100$ mM are described well by a model that assumes a single binding site for the cytochrome on the peroxidase, while data collected at μ = 50 mM require a model that assumes the presence of two nonequivalent, noninteracting binding sites. The binding parameters derived from these experiments are included in Table 1. From these values, it is clear that under solution conditions where both binding sites can be detected, the affinity of the high-affinity site for ferricytochrome *c* is approximately three orders of magnitude greater than that of the low-affinity site.

To define the properties of the two binding sites observed at low ionic strength further, the effect of pH on the net change in proton binding observed on addition of cytochrome *c* to the peroxidase was studied at μ = 50 mM (Figure 2). From these results, it is apparent that the effect of cytochrome *c* binding on the net change in proton binding varies significantly with pH. For all but the most alkaline pH studied, the binding data are analyzed adequately only by the assumption of two binding sites for the cytochrome on its peroxidase. At pH 7.75, the two-site model describes the general shape of the binding curve, but it exhibits a systematic deviation from the experimental data that suggests the presence of a more complex

Table 1: Binding Parameters for Formation of the Cytochrome c–Cytochrome c Peroxidase Complex Determined from Potentiometric Titrations Performed As Described under Experimental Procedures[a]

pH	μ (μM)	anion	cytochrome c oxidation state	temp (°C)	K_a (M^{-1})	q_a	K_b (M^{-1})	q_b
5.51	50	NO_3^-	oxidized	25	$1.38(6) \times 10^6$	1.169(5)	$<1 \times 10^3$	<–1.2
5.99	50	NO_3^-	oxidized	25	$2.9(4) \times 10^6$	0.649(3)	$<1 \times 10^3$	<–0.2
5.99	100	NO_3^-	oxidized	25	$2.46(2) \times 10^5$	0.459(2)		
5.99	100	NO_3^-	oxidized	18	$2.59(8) \times 10^5$	0.475(1)		
5.99	100	NO_3^-	oxidized	10	$2.42(6) \times 10^5$	0.524(1)		
5.99	100	NO_3^-	reduced	25	$1.8(3) \times 10^5$	0.33(1)	$1.0(2) \times 10^3$	–2.4(3)
5.99	100	Cl^-	oxidized	25	$3.5(1) \times 10^5$	0.449(1)		
5.99	100	Cl^-	reduced	25	$1.1(2) \times 10^5$	0.46(4)	$2.8(4) \times 10^3$	–3.0(2)
5.99	150	NO_3^-	oxidized	25	$4.41(4) \times 10^4$	0.341(2)		
5.99	200	NO_3^-	oxidized	25	$1.39(4) \times 10^4$	0.166(1)		
6.50	50	NO_3^-	oxidized	25	$4(2) \times 10^6$	0.229(4)	$1.8(9) \times 10^3$	–0.5(2)
7.00	50	NO_3^-	oxidized	25	*b*	~–0.05	*b*	~0.1
7.75	20	NO_3^-	oxidized	25	$2(1) \times 10^6$	–0.47(3)	$1.1(5) \times 10^3$	8(3)
7.75	50	NO_3^-	oxidized	25	$2(1) \times 10^5$	–0.5(1)	$<1 \times 10^3$	>1.7
7.74	50	NO_3^-	reduced	25	$1.0(9) \times 10^5$	–0.5(2)	$<1 \times 10^3$	>2

[a] The uncertainties in the last significant figure for association constants and q values are shown in parentheses. [b] Signal too small for accurate determination.

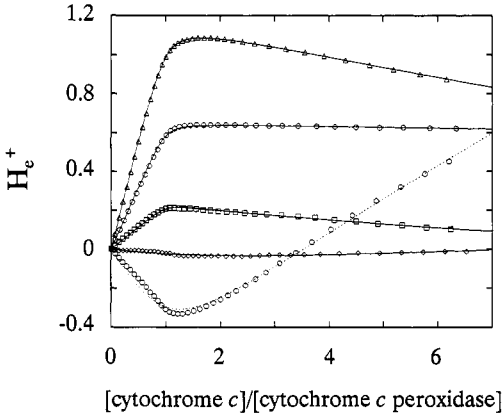


FIGURE 2: Effect of pH on proton release during the titration of cytochrome c peroxidase with ferricytochrome c (μ = 50 mM, 25 °C, KNO_3): (▲) pH = 5.51, $P_{initial}$ = 33.9 μM; (○) pH = 5.99, $P_{initial}$ = 34.3 μM; (□) pH = 6.50, $P_{initial}$ = 36.9 μM; (◇) pH = 7.00, $P_{initial}$ = 37.0 μM; and (○, dashed line) pH = 7.75, $P_{initial}$ = 37.2 μM. The lines are unweighted least-squares fits for a model with two binding sites for cytochrome c per molecule of peroxidase.

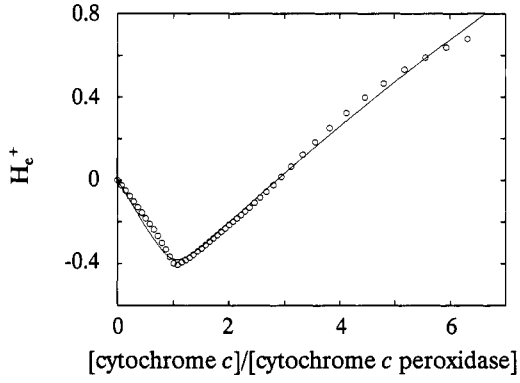


FIGURE 3: Titration of cytochrome c peroxidase with ferricytochrome c (μ = 20 mM, KNO_3, 25 °C, pH 7.75, $P_{initial}$ = 35.5 μM). The curve represents the unweighted least-squares fit of the data to the model for two noninteracting cytochrome c binding sites per cytochrome c peroxidase molecule.

binding interaction. This systematic deviation in the fit for the two-site model is also observed at even lower ionic strength (μ = 20 mM) at pH 7.75 (Figure 3).

The pH dependence of the affinity constants for cytochrome c binding to the high-affinity site is shown in Figure 4A. The values for log K_a shown in Figure 4A were calculated from the measured q values, the value of K_a at pH 6.0, and the

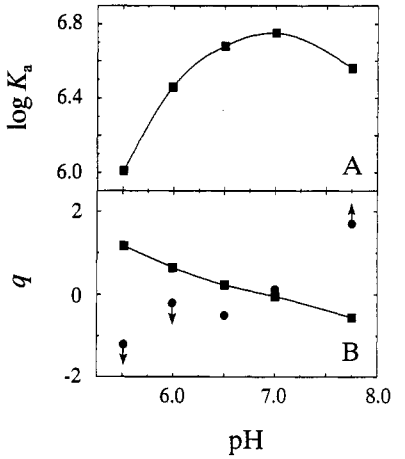


FIGURE 4: pH dependence of q and log K_a for the association of ferricytochrome c with cytochrome c peroxidase (μ = 50 mM, KNO_3, 25 °C). (A) Dependence of the affinity of the high-affinity binding site for cytochrome c on pH. (B) Variation in mole fraction of protons exchanged at the high-affinity (■) and low-affinity (●) binding sites. The symbols at the base of the arrows indicate the maximum or minimum values for the parameters plotted. The lines have no statistical significance.

relationship of pH and association constant derived previously (Edsall & Wyman, 1958; Laskowski & Finkenstadt, 1972):

$$(\log K)_{pH_2} = (\log K)_{pH_1} + \int_{pH_1}^{pH_2} q \, d(pH) \qquad (9)$$

In our experiments, data were collected to high molar ratios of cytochrome c relative to the peroxidase, thereby allowing more accurate determination of q values. The values of log K_a calculated from these q values agree well with those determined from the least-squares fits to the titration curves (Table 1) except at pH 7.75. At this pH, fits to the two-site model deviated significantly from the experimental data at low molar ratios, thereby suggesting that log K_a of 6.56 calculated from the q_a value may be a more accurate measure of the binding affinity than the value provided in Table 1. Although limits for the stability of complex formation at the low-affinity site could be estimated (Table 1), these values were of insufficient certainty to permit inclusion in Figure 4A owing to the difficulty of defining the contribution of the low-affinity site to net change in proton binding in the presence of a dominant high-affinity site. In cases of very weak binding, the titrations produce smooth curves that make it difficult to assess the values q and K, which are highly correlated

(Laskowski & Finkenstadt, 1972). In such cases, the data can frequently be fitted adequately by a broad range of (q_b, K_b) values. As can be seen, optimum complex stability for the high-affinity site occurs between pH 6.75 and 7.

The corresponding dependence of the mole fraction of protons exchanged at the high- and low-affinity binding sites is shown in Figure 4B. In this case, the values derived for the low-affinity binding site are sufficiently defined that reasonable limits can be included in this plot for qualitative comparisons. From the resulting figure, it is apparent that the slopes of the q vs pH plots for the high- and low-affinity binding sites are of opposite sign. Interestingly, at most values of pH, binding of cytochrome c to one site results in proton release while binding of the cytochrome to the other site results in proton uptake. Thus, the two binding sites exhibit distinctly different electrostatic properties apart from the considerable difference in binding affinity that they exhibit. In the dependence of q on pH, $q = 0$ at the pH where complex stability is maximal or minimal. The apparent intersection of the q vs pH plots for the two binding sites near $q = 0$ leads us to the tentative conclusion that the optimum pH for binding at the high-affinity site occurs at this pH (pH 6.75–7) while the binding of the cytochrome to the low-affinity site is least stable at the same pH. The uncertainty in the q_b values derived from our data prevents a more definitive analysis of this relationship.

The dependence of complex formation on temperature was investigated by studying complex formation at three different temperatures. From the results shown in Table 1, the binding parameters determined in these experiments were essentially independent of temperature. Although potentiometric data were obtained at just three temperatures, we can estimate interim values for $\Delta H° = 0.2 \pm 0.8$ kcal/mol and $\Delta S° = 26 \pm 3$ eu for the high-affinity site from these data. These estimates indicate that the interaction between these two proteins is largely entropic in nature as previously observed for the cytochrome c–cytochrome b_5 complex (Mauk et al., 1984). The extent of surface desolvation or retention of surface solvent molecules remains to be evaluated in subsequent studies employing other techniques.

The effects of cytochrome c oxidation state and solution anion composition on the stability of complex formation were also studied as these factors have been argued in the past to exert considerable influence on the stability of cytochrome c–cytochrome peroxidase complex formation (Hake et al., 1992). Our results indicate that the reduced cytochrome binds with a somewhat lower affinity at the high-affinity site than does the oxidized cytochrome (Figure 5 and Table 1). Interestingly, however, we were able to detect binding of the reduced cytochrome to the low-affinity site at relatively high ionic strength (100 mM) at pH 6 while we were unable to detect binding of the ferricytochrome at this site under these conditions. This observation is presumably significant because we are unable to detect binding of the ferricytochrome to the low-affinity site at this high ionic strength at any pH (Table 1). In addition, our results indicate that the anion composition of the solution has relatively little effect on the stability of complex formation (Figure 5 and Table 1).

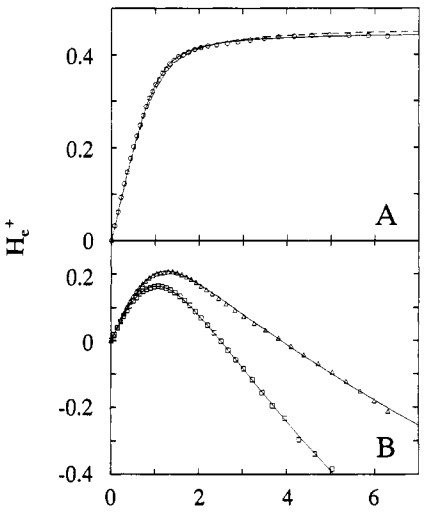


FIGURE 5: Effect of medium composition and cytochrome c oxidation state on the association of cytochrome c with cytochrome c peroxidase. (A) Effect of Cl^- vs NO_3^- on the titration of the peroxidase with ferricytochrome c: (O with solid line) pH = 5.99, μ = 100 mM in KCl, 25 °C, $P_{initial}$ = 36.6 μM. The solid line is the unweighted least-squares fit assuming a model allowing one cytochrome binding site per peroxidase molecule. The result employing the same experimental conditions except with KNO_3^- replacing KCl is shown by the dashed line. This dashed line is a simulation using parameters determined experimentally to be $K_a = 2.46 \times 10^5$ M^{-1} and $q_a = 0.459$ (see Table 1). (B) Association of cytochrome c peroxidase and ferrocytochrome c (pH 5.99, μ = 100 mM, 25 °C): (Δ) in KNO_3, $P_{initial}$ = 58.7 μM; (□) in KCl, $P_{initial}$ = 38.7 μM. Both lines are unweighted least-squares fits for the model with two cytochrome c binding sites per peroxidase molecule.

DISCUSSION

In the present study, we have assessed the electrostatic properties of the interaction between cytochrome c and cytochrome c peroxidase by a potentiometric method that provides direct measurement of the net change in protonation caused by the interaction of two proteins. This technique provides unique mechanistic information concerning the manner in which these proteins interact, and it provides a sensitive and precise method of quantifying the stability of complex formation. In a similar analysis of the interaction of cytochrome c with cytochrome b_5, we were also able to measure the complexation-induced difference titration curve directly, which in turn allowed us to derive a continuous measure of the variation in association constant with pH. This latter type of experiment was not feasible in the current study because the presence of a second, low-affinity binding site complicates the experimental data sufficiently that a meaningful numerical analysis cannot be performed.

The results reported here establish that the interaction of cytochrome c with cytochrome c peroxidase is characterized by the binding of the cytochrome to two functionally different sites on cytochrome c peroxidase in a pH-linked fashion. Previous studies of functional properties of this complex at low ionic strength have generally employed either pH 6 or 7 with the tacit assumption that complex stability is comparable under both conditions. While this assumption may be adequate for some purposes, the current results (Figure 3) demonstrate that the cytochrome binds to the high-affinity site with slightly greater affinity at pH 7 than it does at pH 6. Perhaps more importantly, the protonation status of the cytochrome–peroxidase complex is clearly different at the two values of pH, so the structure of the complex varies with pH in a manner that remains to be characterized in detail.

The stoichiometry of cytochrome c–cytochrome c peroxidase complex formation has been a subject of continuing debate for several years. The first indication that the peroxidase might possess two binding sites for the cytochrome was provided by kinetic and gel filtration experiments (Kang et al., 1977). Several subsequent reports involving steady-state fluorescence quenching titrations (Vitello & Erman, 1987),

analytical ultracentrifugation (Dowe et al., 1984), and electronic difference spectroscopy (Erman & Vitello, 1980) led to the conclusion that only a single binding site exists, although an alternative strategy for fluorescence quenching titrations suggested the presence of a second site (Kornblatt & English, 1987), and more recent and more extensive fluorescence and electron transfer kinetics experiments provide further support for this conclusion (Stemp & Hoffman, 1993; Zhou & Hoffman, 1993, 1994). These latter studies indicate that at low ionic strength and at cytochrome *c*/cytochrome *c* peroxidase ratios >1, a second cytochrome *c* binding site that is active in electron transfer can be detected. More recently, Yi et al. (1994) have reported NMR studies of the complex that can also be interpreted in terms of two binding sites for the cytochrome. Our potentiometric measurements permit the direct detection of both binding sites with a sensitivity and precision that have not been possible with techniques previously employed. By studying the interaction of these two proteins over a wide range of solution conditions, the basis for the apparent discrepancies in stoichiometry determined in previous studies can be at least partially explained. Probably the principal difficulty in detecting two binding sites in this system is the great difference in binding affinity exhibited by the two sites; detection of the low-affinity site in the presence of the high-affinity site simply requires the use of techniques that are capable of detecting the low-affinity site under this circumstance. Even with highly sensitive techniques, the low-affinity site may be of sufficiently low affinity that it escapes detection under some solution conditions.

Our finding that the oxidation state of cytochrome *c* has a small effect on the stability of the complex formed with its peroxidase is consistent with previous fluorescence titration studies of Vitello and Erman (1987) in which the fluorescence quenching of porphyrin cytochrome *c* peroxidase by ferri- and ferrocytochrome *c* was compared. Both of these studies, however, differ from the recent report by Hake et al. (1992) in which gel chromatography techniques were used to demonstrate that the reduced cytochrome binds to the peroxidase with far greater stability than the oxidized protein. From a structural or electrostatic perspective, there is no obvious mechanistic reason to expect that the oxidation state of the cytochrome should cause a significant difference in complex stability. The oxidation-state-linked conformation change of cytochrome *c* is quite small and causes no perceptible structural change at the surface of the protein that should affect the interaction of these two proteins. Second, the net electrostatic charge of ferrocytochrome *c* has one less positive charge than does the ferricytochrome. This fact alone leads to the expectation that the oxidized protein should have a slightly greater affinity for the peroxidase than that of the reduced cytochrome as we observe. For these reasons, we are inclined to explain the difference in results obtained by Hake et al. (1992) and those obtained in the current study and in the work of Vitello and Erman (1987) as arising from subtle but significant complications in the use of chromatographic techniques, particularly with the types of proteins and elution conditions required with systems of this type.

The location of the high-affinity cytochrome binding site on the surface of the peroxidase is presumably identical or at least closely related to that defined by the crystallographic study of Pelletier and Kraut (1992) although some recent solution studies seem to indicate that this binding site is better described as a binding domain containing several overlapping sites (McLendon et al., 1993; Stemp & Hoffman, 1993) giving rise to several similar complexes that interconvert rapidly at room temperature. Quantitative comparison of the potentiometric results with the behavior implied for the high-affinity site by the crystallographically determined structure will require detailed electrostatic modeling calculations. The location of the low-affinity binding site, on the other hand, is conjectural. At present, the only available indication of the nature of this site is provided by Brownian dynamics simulations of cytochrome *c*–cytochrome *c* peroxidase encounter complex formation reported by Northrup et al. (1988). From examination of the secondary encounter site near Asp150 on the surface of the peroxidase predicted by these simulations, it is not immediately apparent why the binding of the cytochrome to this site should be a thousand-fold less stable than to the primary encounter site. However, in considering the properties of the low affinity site it is undoubtedly important to consider also the electrostatic and (to a lesser extent) the steric consequences of having the high-affinity site occupied by a molecule of cytochrome *c* on the properties of the low-affinity site (Thomasson & Northrup, 1992).

Stemp and Hoffman (1993) have reported that cytochrome *c* binding to the high affinity site of heme-substituted fluorescent peroxidase derivatives results in highly efficient energy transfer and less efficient electron transfer between the heme centers of the two proteins, while cytochrome *c* bound at the low-affinity site is much more efficient in electron transfer than energy transfer. This functional distinction between these two sites is now complemented by our demonstration that the electrostatics of cytochrome binding to the two sites is also distinctly different as described in the previous section. In light of the increasing evidence supporting the existence of and defining the properties of the low-affinity binding site, the acquisition of experimental information concerning the location of this site is increasingly important. As indicated above, the difficulties resulting from the necessity of studying this site in the presence of the high-affinity site will make this quest particularly challenging.

ACKNOWLEDGMENT

We thank Drs. Mathew Mauro and Thomas Poulos for consultation regarding purification of recombinant cytochrome *c* peroxidase, Drs. James Erman and Lidia Vitello for a sample of cytochrome *c* peroxidase isolated from commercial yeast, and Dr. Scott Northrup for helpful discussions.

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