Electron Transfer Complexes of Cytochrome *c* Peroxidase from *Paracoccus denitrificans* Containing More than One Cytochrome[†]

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Received May 19, 2003; Revised Manuscript Received August 21, 2003

ABSTRACT: According to the model proposed in previous papers [Pettigrew, G. W., Prazeres, S., Costa, C., Palma, N., Krippahl, L., and Moura, J. J. (1999) The structure of an electron-transfer complex containing a cytochrome *c* and a peroxidase, *J. Biol. Chem. 274*, 11383−11389; Pettigrew, G. W., Goodhew, C. F., Cooper, A., Nutley, M., Jumel, K., and Harding, S. E. (2003) Electron transfer complexes of cytochrome *c* peroxidase from *Paracoccus denitrificans*, *Biochemistry 42*, 2046−2055], cytochrome *c* peroxidase of *Paracoccus denitrificans* can accommodate horse cytochrome *c* and *Paracoccus* cytochrome c_{550} at different sites on its molecular surface. Here we use ^{1}H NMR spectroscopy, analytical ultracentrifugation, molecular docking simulation, and microcalorimetry to investigate whether these small cytochromes can be accommodated simultaneously in the formation of a ternary complex. The pattern of perturbation of heme methyl and methionine methyl resonances in binary and ternary solutions shows that a ternary complex can be formed, and this is confirmed by the increase in the sedimentation coefficient upon addition of horse cytochrome *c* to a solution in which cytochrome c_{550} fully occupies its binding site on cytochrome *c* peroxidase. Docking experiments in which favored binary solutions of cytochrome c_{550} bound to cytochrome *c* peroxidase act as targets for horse cytochrome *c* and the reciprocal experiments in which favored binary solutions of horse cytochrome *c* bound to cytochrome *c* peroxidase act as targets for cytochrome c_{550} show that the enzyme can accommodate both cytochromes at the same time on adjacent sites. Microcalorimetric titrations are difficult to interpret but are consistent with a weakened binding of horse cytochrome *c* to a binary complex of cytochrome *c* peroxidase and cytochrome c_{550} and binding of cytochrome c_{550} to the cytochrome *c* peroxidase that is affected little by the presence of horse cytochrome *c* in the other site. The presence of a substantial capture surface for small cytochromes on the cytochrome *c* peroxidase has implications for rate enhancement mechanisms which ensure that the two electrons required for re-reduction of the enzyme after reaction with hydrogen peroxide are delivered efficiently.

The original concept of a lock-and-key relationship between redox partners in an electron transfer complex has given way to a more complex picture of a family of weakly bound orientations of one protein against the other during an encounter (*1*, *2*). For one of the best studied electron transfer systems, that of yeast cytochrome *c* peroxidase, a crystallographic solution has been obtained for the enzyme in association with a cytochrome *c* (*3*). It may be that this solution is just one of several possible orientations during encounter, and this underlines the problem of studying systems that form weak complexes. The existence of secondary sites on redox enzymes has been a recurring theme in work on electron transfer complexes. An example is the secondary site on yeast cytochrome *c* peroxidase which was originally controversial but is now accepted as a structurally distinct weaker binding site (*4*). It is often not clear whether a secondary site is a functional electron transfer site or a site which allows a second redox protein to be waiting ready to switch position when the primary site is vacated. One can see the potential advantages of multiple electron transfer sites when the redox process catalyzed by the enzyme requires more than one electron. Rapid synchronous delivery of electrons may protect against the formation and release of partly reduced chemical species which have toxic radical character. A similar argument may apply to binding sites which are not electron transfer sites. These may be part of a general capture surface which facilitates rate enhancement by reducing the dimensions of search. A second redox protein may be held at such a site so that it can readily move into the electron transfer site when it is vacated and ensure rapid delivery of a second electron. In the case of yeast cytochrome

[†] G.W.P. acknowledges the support of BBSRC (Grant 15B/13005). The microcalorimetry facility (University of Glasgow) and the analytical ultracentrifugation facility (University of Nottingham) are funded jointly by the BBSRC and EPSRC. S.R.P. thanks the Fundacao para a Ciencia e Tecnologia (BD/18297/98) for its support.

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10.1021/bi034829c CCC: $25.00
Published on Web 09/26/2003

c peroxidase, Mei et al. (*5*) have shown that the second site is not functional in electron transfer and propose that it acts to promote dissociation of the product cytochrome *c* from the electron transfer site.

Our model system for studying such questions has been the diheme cytochrome *c* peroxidase from *Paracoccus denitrificans* and the complexes that this enzyme forms with its redox partners. After reaction with hydrogen peroxide, the oxidized enzyme requires two electrons to return it to an active form in which one heme (the electron-transferring or E heme) contains ferrous iron and the other heme (the peroxidatic or P heme) contains ferric iron.

A small mono-heme class I cytochrome *c* called cytochrome c_{550} acts as a physiological donor to the cytochrome *c* peroxidase. This cytochrome is a homologue of mitochondrial cytochromes *c*, and indeed, horse cytochrome *c* acts as a nonphysiological electron donor that has kinetic activity similar to that of cytochrome c_{550}. We have used the combination of the physiological and nonphysiological redox partners to probe the nature of the complexes formed with the cytochrome *c* peroxidase. This sort of approach has also been a feature of studies of the monoheme yeast cytochrome *c* peroxidase (*6*, *7*).

Using a combination of molecular docking simulation and ^{1}H NMR spectroscopy, we have shown that horse cytochrome *c* and cytochrome c_{550} bind to the cytochrome *c* peroxidase at distinct surface sites (*8*). The cytochrome c_{550} is located above the exposed edge of the E heme, and the horse cytochrome *c* is located on the surface roughly equidistant between the E and P heme groups. The location and orientation of cytochrome c_{550} are exactly what might have been predicted for an electron transfer complex. Its heme edge is almost touching that of the E heme, and the iron−iron distance is 16 Å. Whether the preferred "between hemes" site for horse cytochrome is also an electron transfer site is more debatable since the iron−iron distances are 22−23 Å.

The binding of both cytochromes is endothermic and driven by a large entropy change, consistent with a freeing of water molecules from the interface (*9*). The enzyme binds just one cytochrome c_{550} but can accommodate two horse cytochrome *c* molecules in higher- and lower-affinity binding sites. The binding to the higher-affinity site is weakened by increasing the ionic strength in the range of 0.01−0.05 M, and this is accompanied by an increase in the steady state oxidation rates (*9*). If we accept that the high-affinity site equates with the between hemes site observed for horse cytochrome *c* in the molecular docking simulation, we can propose that this represents a nonproductive orientation which is released by increasing the ionic strength. According to this model, the lower-affinity site for horse cytochrome *c* may be equated with the E heme site, and indeed, if the top-ranking binary complex of horse cytochrome *c* and cytochrome *c* peroxidase is used as a target for ternary complex formation with a second horse cytochrome *c*, the preferred binding is at the E heme (*9*).

In this study, we investigate whether the cytochrome *c* peroxidase can accommodate both cytochrome c_{550} and horse cytochrome *c* at the same time in a ternary complex. Demonstration of such a complex would tend to support the current model for how the two cytochromes bind, but it would also raise the possibility of an electron transfer mechanism which ensures the synchronous or rapid sequential delivery of two electrons.

MATERIALS AND METHODS

Source of Proteins. Cytochrome *c* peroxidase and cytochrome c_{550} were purified from *P. denitrificans* [ATCC 19367, NCIB 8944, LMD 52.44) (now called *Paracoccus pantotrophus* (*10*)] as described by Goodhew et al. (*11*) and used in the oxidized state. Horse cytochrome *c* (C7752) was obtained from Sigma and used in the oxidized state. Enzyme and cytochrome concentrations were determined using extinction coefficients of 250 mM^{-1} cm^{-1} (cytochrome *c* peroxidase, 409 nm), 108 mM^{-1} cm^{-1} (cytochrome c_{550}, 410 nm), and 109 mM^{-1} cm^{-1} (horse cytochrome *c*, 408 nm).

^{1}H NMR Spectroscopy. High-resolution ^{1}H NMR spectra were recorded in the Fourier transform mode on a Bruker ARX-400 spectrometer (400 MHz). Data were recorded at 299 K, and 2000 transients were acquired for each spectrum. To improve the signal-to-noise ratio, an exponential multiplication by 10 Hz line broadening of the free induction decay was applied prior to Fourier transformation. All chemical shifts are quoted in parts per million (ppm) from internal 3-trimethylsilyl[2,2,3,3-$^2H_4$]propionate; positive values refer to low-field shifts. Protein samples were exchanged several times with the appropriate buffer in D_2O by centrifugation above a Vivaspin membrane. (Note that the spectra iii and iv of Figure 1 do not appear to reflect the equimolar composition of horse cytochrome *c* and cytochrome c_{550}. In part, this is due to the dramatic broadening effect on the heme methyls of the cytochrome c_{550} when bound to the peroxidase, making integration unreliable. If spectrum iv of Figure 1 is compared to Figure 6A of ref *8*, it is clear that in the presence of NaCl, the complex has not fully dissociated and the heme methyls of cytochrome c_{550} remain quite broad. In addition, the heme methyls of horse cytochrome *c* in spectrum iii of Figure 1 lie on top of broad peroxidase resonances making their integration difficult.)

Microcalorimetry. Protein solutions were equilibrated with the appropriate buffer by molecular exclusion chromatography on Sephadex G25. The titrant solution was then concentrated by centrifugation above a Vivaspin membrane (M_r cutoff of 5000). Protein concentrations were determined using the extinction coefficients noted above.

The target cytochrome *c* peroxidase was degassed and placed in the sample chamber of the VP-ITC[1] microcalorimeter (Microcal). The syringe was filled with a solution of the protein used to form the binary complex, and successive injections of 10 μL were delivered into the chamber while its contents were stirred (310 rpm). The duration of the additions was 20 s, and they were 180 s apart. At a chosen point of particular proportions of probe protein to target, the syringe was refilled with the other protein used to form the ternary complex. Heat changes were analyzed within Microcal Origin software which fits on the basis of iteration within a Marquandt routine. Data were fitted using models for one set of sites or for two independent sets of sites. Dilution titrations of the probe protein into buffer alone

[1] Abbreviations: ccp, cytochrome *c* peroxidase; Mes, 2-(*N*-morpholino)ethanesulfonic acid; ITC, isothermal titration calorimetry; *D*, diffusion coefficient; *s*, sedimentation coefficient; $\Delta H°$, standard enthalpy change.

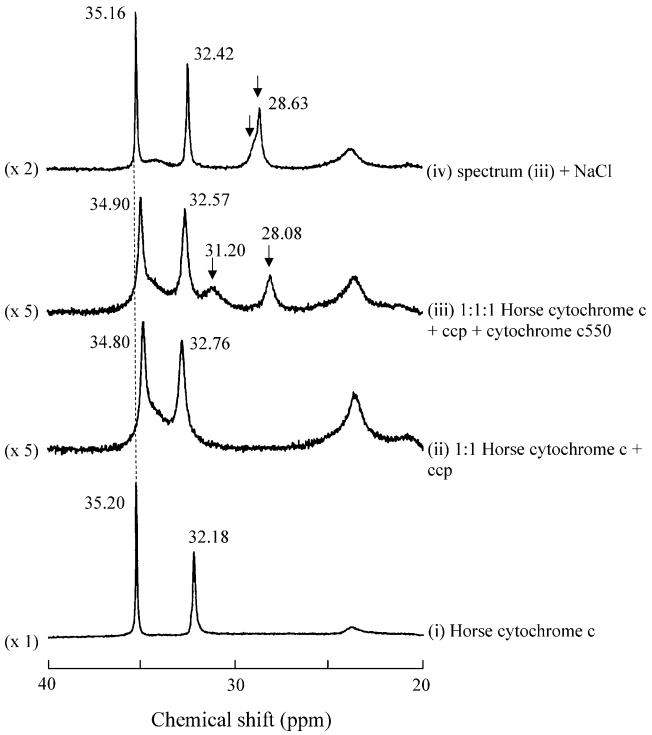

FIGURE 1: ^{1}H NMR spectroscopy of the complex formed between horse cytochrome *c*, cytochrome c_{550}, and cytochrome *c* peroxidase. Shown is the downfield heme methyl region. To a solution (0.75 mM) of horse cytochrome *c* in 10 mM Mes, 25 mM NaCl (pH 6.0), and D_2O (i) was added equimolar cytochrome *c* peroxidase from a 3.34 mM stock solution in the same buffer (ii) (final concentration of 612 μM). Then equimolar cytochrome c_{550} from a 4.18 mM stock solution in the same buffer was added (iii) (final concentration of 536 μM). Finally, the solution in the NMR tube containing all three proteins was taken to 700 mM NaCl from a 3 M stock solution in the same buffer (iv) (final concentration of 411 μM). The dashed line indicates the free position of heme methyl 8 of the horse cytochrome *c*. The arrows in spectra iii and iv denote the heme methyl peaks of the cytochrome c_{550}. In the free state, these methyls have chemical shifts of 29.1 and 28.7 ppm (not shown). The former shifts to 31.20 ppm and the latter to 28.08 on binding the peroxidase (iii). They return to near their free positions in the presence of 700 mM NaCl (iv).

were also performed and the results used to correct the binding titrations.

Analytical Ultracentrifugation. Partial specific volumes of the proteins were calculated from the amino acid compositions and are 0.732 mL/g for cytochrome *c* peroxidase, 0.735 mL/g for cytochrome c_{550}, and 0.744 mL/g for horse cytochrome *c*. Protein solutions were equilibrated with the appropriate buffer by molecular exclusion chromatography on Sephadex G25 and were used at a concentration ranging from 5 to 40 μM. Nonideality effects were assumed to be negligible at the dilute concentrations that were studied.

The Beckman Optima XL-A or XL-I (Beckman, Palo Alto) analytical ultracentrifuges, equipped with scanning absorption optics, were used in all investigations. Sedimentation velocity experiments were carried out at 45 000 rpm and 25 °C. Sedimentation coefficients were obtained by scanning at 440 nm. The extinction coefficients of cytochrome *c* peroxidase, horse cytochrome *c*, and cytochrome c_{550} at these wavelengths are 33, 17.8, and 15 mM^{-1} cm^{-1}, respectively. The DCDT+ program of Philo (*12*) was used to analyze groups of boundaries to derive sedimentation coefficients. This method is based on the time-derivative method developed by Stafford (*13*, *14*) which fits Gaussian functions to the so-called $g(s^*)$ distribution. For a given component, the breadth of this distribution also facilitates an estimate for the translational diffusion coefficient D^*. The asterisks indicate the inability to distinguish whether intensity on either side of a peak really represents material sedimenting with that *s* value or is merely broadening due to diffusion of material sedimenting at the *s* value of the peak. Sedimentation and diffusion coefficients were corrected to standard solvent conditions (the viscosity, and the density of water at 20 °C) using the SEDNTERP program (based on ref *15*). The derivation of a value of M_r from the sedimentation velocity experiments is based on the s/D ratio and is dependent on reliable estimates of both. In the case of interacting systems such as those described here, a boundary may contain more than one species in equilibrium, and M_r values derived from such a boundary can only be regarded as approximate estimates. In such a case, the diffusion coefficient is likely to be an overestimate and the M_r an underestimate.

Individual sedimentation velocity scans were used to calculate the binding of a cytochrome *c* to the peroxidase. For a given total cytochrome *c*, the proportion bound to the cytochrome *c* peroxidase could be calculated from the increment in the absorbance of the leading boundary over that due to the peroxidase itself. The proportion of free cytochrome could be calculated from the absorbance at the trailing boundary. When the separation between the leading and trailing boundaries was difficult to resolve in a single scan, the absorbance of the trailing boundary was obtained from a later scan in which the leading boundary had already sedimented. The absorbance values of boundaries were corrected for the radial dilution effect of the sectored cell.

Molecular Docking. Docking was performed as described in ref *8* using the algorithm BiGGER developed by Palma et al. (*16*). The original algorithm performs a complete and systematic search of the rotational space of one protein relative to the other, generating a large number of docking geometries based solely on the complementarity of the molecular surfaces. The 1000 best solutions thus generated were finally evaluated and ranked according to a combination of additional interaction criteria that include the electrostatic energy of interaction, the relative solvation energy, and the relative propensity of adjacent side chains to interact. For each solution, this combination process produces a "global score". This original algorithm has evolved through several optimizing stages (see http://www.dq.fct.unl.pt/bioin/chemera) to allow "softer" docking in which side chains are truncated. This allows greater overlap between the structures by ignoring steric clashes of the more flexible side chains. As a consequence, correct solutions are more reliably captured in some model cases, but a higher level of "noise" is introduced due to the retention of spurious incorrect solutions. To partly counteract that, there is a second filtering involving evaluation of the likelihood of side chain contacts. This evaluation is based on a set of 87 structures used to train the neural network classifier.

"Soft" and "hard" docking give different results. If the conformations of the docking partners do not change significantly, the hard docking algorithm (being more

stringent) is better at filtering out "incorrect" models at the stage of the surface complementarity calculations. However, if there are critical changes in the side chain conformation, the hard docking may reject correct models due to excessive overlap and only the soft docking has a chance of retaining these. In the general case, with no additional information available, the use of soft docking is preferred to improve the probability of retaining at least one "correct" model at the stage of the surface complementarity filter. But if additional information (such as electron-transfer constraints) is available, one can confront the results of soft and hard docking and ask whether the former retains "likely" models that the latter fails to do. If that is not the case, then the hard docking results can be preferred.

RESULTS

Detection of Ternary Complex Formation by ^1H NMR Spectroscopy. Both cytochrome c_{550} and horse cytochrome *c* have two strongly downfield shifted heme methyl groups which are further shifted on binding cytochrome *c* peroxidase (*8*). The progressive shift in the resonances during these titrations suggests a fast exchange, and the observed substantial broadening is probably due to the much higher molecular weight of the complexes (an addition of an M_r of 75 000 for the peroxidase dimer) relative to the cytochromes ($M_r = 12000-15000$). This broadening and the shift effect are seen for horse cytochrome *c* in spectrum ii of Figure 1 and involve a 0.4 ppm upfield shift in a heme methyl from 35.20 to 34.80 ppm. Addition of equimolar cytochrome c_{550} to the horse cytochrome *c*−peroxidase complex results in a small shift back (0.1 ppm) toward the 35.20 ppm position (spectrum iii of Figure 1), and the "free" position can be almost fully restored by addition of a high NaCl concentration (spectrum iv of Figure 1). The magnitudes of the shifts suggest that perhaps 25% of the horse cytochrome *c* is displaced by the addition of the cytochrome c_{550}. The second heme methyl of the horse cytochrome *c* (32.18 ppm) is also shifted (this time further downfield to 32.76 ppm) by the binding to the peroxidase (spectrum ii of Figure 1), and again this shift survives addition of the cytochrome c_{550} (spectrum iii of Figure 1). In this case, however, the original free peak position is not restored by addition of a high concentration of NaCl (spectrum iv of Figure 1). This is due to an effect of the NaCl on the position of this methyl in the absence of binding cytochromes (data not shown).

At the same time, it is clear that the cytochrome c_{550} itself is bound. The chemical shifts of the two heme methyls of free cytochrome c_{550} are at 29.1 and 28.7 ppm (*8*). The former methyl shifts to 31.2 ppm and the latter to 28.1 on binding the peroxidase (*8*). These shifts are clearly seen in spectrum iii of Figure 1. Again the association with cytochrome c_{550} can be broken by a high NaCl concentration with the two heme methyls coming together near 28.63 ppm (spectrum iv of Figure 1).

Support for the existence of a ternary complex also comes from an examination of the upfield region populated by the resonances associated with the methionine ligands of the cytochrome hemes. The methionine methyl of free cytochrome c_{550} has a chemical shift of −17.87 ppm (spectrum i of Figure 2A), and this is shifted downfield by 0.8 ppm in the presence of cytochrome *c* peroxidase (*8*). The same shift

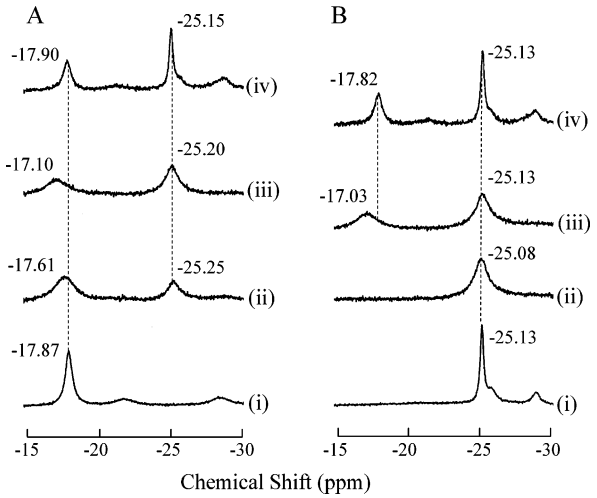

FIGURE 2: ^1H NMR spectroscopy of the complex formed between horse cytochrome *c*, cytochrome c_{550}, and cytochrome *c* peroxidase. Shown is the upfield methionine methyl region. Panel A contains spectra from an experiment in which the NMR tube initially contained 0.75 mM cytochrome c_{550} in 10 mM Mes, 25 mM NaCl (pH 6.0), and D_2O (i) to which was added an equimolar amount of a horse cytochrome *c*/cytochrome *c* peroxidase solution from a stock in which each protein is at 1.2 mM (iii) (final concentration of 462 μM). Then the solution in the NMR tube containing all three proteins was taken to 700 mM NaCl from a 3 M stock solution in the same buffer (iv) (final concentration of 362 μM). Spectrum ii was obtained after addition of a half-molar amount of the binary complex of horse cytochrome *c* and cytochrome *c* peroxidase. The dashed line at −25.15 ppm denotes the virtually constant position of the methionine methyl of horse cytochrome *c*. The dashed line at −17.87 ppm denotes the free position of the methionine methyl of cytochrome c_{550}. Panel B shows spectra obtained during the same experiment as shown in Figure 1. Final concentrations are like those for Figure 1. To a solution (0.75 mM) of horse cytochrome *c* in 10 mM Mes, 25 mM NaCl (pH 6.0), and D_2O (i) was added an equimolar amount of cytochrome *c* peroxidase from a 3.34 mM stock solution in the same buffer (ii) and then an equimolar amount of cytochrome c_{550} from a 4.18 mM stock solution in the same buffer (iii). Finally, the solution in the NMR tube containing all three proteins was taken to 700 mM NaCl from a 3 M stock solution in the same buffer (iv). The dashed line at −25.13 ppm denotes the virtually constant position of the methionine methyl of horse cytochrome *c*. The dashed line at −17.82 ppm denotes the free position of the methionine methyl of cytochrome c_{550} in the presence of added NaCl. In both panels A and B, the initial spectrum (i) is unmagnified and the others are magnified 2-fold.

is observed when an equimolar complex of horse cytochrome *c* and cytochrome *c* peroxidase is added to cytochrome c_{550} (spectrum iii of Figure 2A), and the cytochrome c_{550} is released by addition of NaCl (spectrum iv of Figure 2A). This effect is also seen in the inverse experiment when cytochrome c_{550} is added to the equimolar complex of horse cytochrome *c* and cytochrome *c* peroxidase (spectrum iii of Figure 2B). Again, the cytochrome c_{550} is released on addition of a high NaCl concentration (spectrum iv of Figure 2B). The methionine methyl of horse cytochrome *c* at −25.13 ppm is scarcely shifted upon binding to the cytochrome *c* peroxidase, but it does broaden due to the increase in the associated particle size (spectrum ii of Figure 2B). This broadened state is maintained in the presence of cytochrome c_{550} (spectrum iii of Figure 2A and spectrum iii of Figure 2B), and the peak sharpens again on addition of NaCl (spectrum iv of Figure 2A and spectrum iv of Figure 2B). Thus, in spectrum iii of Figure 2A and spectrum iii of Figure 2B, we have evidence that a ternary complex between

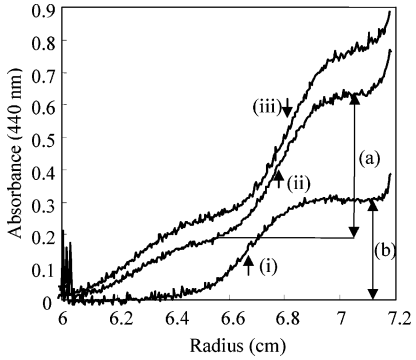

FIGURE 3: Sedimentation velocity boundaries of *Paracoccus* cytochrome *c* peroxidase alone and in the presence of binding cytochromes. Solutions of cytochrome *c* peroxidase alone (10 μM) (i) or in the presence of 20 μM cytochrome c_{550} (ii) or 20 μM cytochrome c_{550} and 10 μM horse cytochrome *c* (iii) were centrifuged at 45 000 rpm in 10 mM Mes, 10 mM NaCl, and 2 mM $CaCl_2$ at pH 6 and 25 °C. Absorbance scans of the centrifuge cells were carried out at 440 nm. The scans shown here are taken at equivalent time points for the three experiments. The arrows indicate the midpoints of the leading boundary. *a* is the absorbance of the leading boundary containing cytochrome c_{550} and cytochrome *c* peroxidase; *b* is the absorbance due to the cytochrome *c* peroxidase alone.

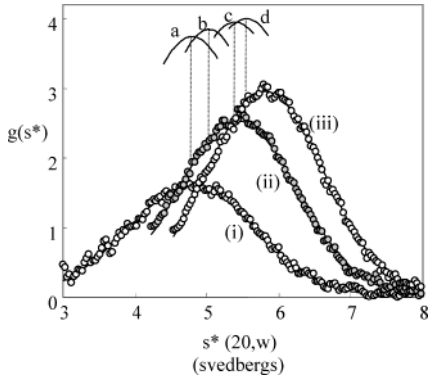

FIGURE 4: Effect of cytochrome *c* binding on the sedimentation coefficient of the *Paracoccus* cytochrome *c* peroxidase. Sedimentation velocity experiments were performed in 10 mM Mes, 10 mM NaCl, and 2 mM $CaCl_2$ at pH 6.0, 25 °C, and 45 000 rpm. Paired scans within a set of 10 sequential scans were used to produce a distribution of sedimentation coefficients using the DCDT+ method of Philo (*12*) and refs *13* and *14*. For experiments in which two boundaries were evident, both were analyzed: (i) 10 μM cytochrome *c* peroxidase, (ii) 10 μM cytochrome *c* peroxidase and 20 μM cytochrome c_{550}, and (iii) 10 μM cytochrome *c* peroxidase, 20 μM cytochrome c_{550}, and 10 μM horse cytochrome *c*. Curves a−d are portions of the theoretical fits to experiments in which the proportions of cytochrome c_{550} to cytochrome *c* peroxidase were 0.5:1, 1:1, 2:1, and 4:1, respectively. The vertical axis is in absorbance units per svedberg.

cytochrome *c* peroxidase, cytochrome c_{550}, and horse cytochrome *c* has been formed.

For cytochrome c_{550}, the shifts in heme methyls and the methionine methyl associated with ternary complex formation are the same as those observed for saturation of binding during binary complex formation (*8*), indicating that the binding site is also fully occupied in the ternary complex.

A control experiment was performed in which cytochrome c_{550} is titrated into a horse cytochrome *c* solution. When this is done in 10 mM Mes (pH 6.0) and D_2O, some very small chemical shifts are observed, consistent with weak nonspecific binding (results not shown). One of the reasons for conducting the above experiments in the additional presence of 25 mM NaCl (see the legend of Figure 1) is that these small shifts disappear under these conditions.

Analytical Centrifugation of the Binary and Ternary Complexes. The leading boundaries in Figure 3 represent cytochrome *c* peroxidase (i) or cytochrome *c* peroxidase complexed to cytochromes (ii and iii). The trailing boundaries represent free cytochrome. In the presence of cytochrome c_{550}, the sedimentation coefficient of the leading boundary is increased (absorbance profile ii of Figure 3), indicating binding of the cytochrome c_{550} and the peroxidase. The proportion of cytochrome c_{550} bound is given by $a - b$ (after correction for the radial dilution of the sectored cell), and the proportion that is free is given by the absorbance of the trailing boundary. From these figures and the total amount of cytochrome c_{550} added, the cytochrome c_{550} binding site is 97% occupied under these conditions.

The leading boundary has a sedimentation coefficient ($s_{20,w}$) of 5.43 S for the experiment in which the proportions of cytochrome c_{550} and cytochrome *c* peroxidase are 2:1 (part ii of Figure 4), and an indication of the fits for different proportions of cytochrome c_{550} and cytochrome *c* peroxidase is given by *a* (0.5:1), *b* (1:1), *c* (2:1), and *d* (4:1). The $s_{20,w}$ value of 5.43 S together with an estimate of *D* from the spread of the leading boundary can be used to derive an M_r value of 96 000. The binding of two cytochrome c_{550} molecules (each with an M_r of 14 815) to a cytochrome *c* peroxidase dimer (M_r of 75 026) should yield a particle with an M_r of 104 656. Given that the analysis of boundary absorbances suggests virtually complete occupancy of the cytochrome c_{550} site under these experimental conditions, the underestimate of the molecular weight can be attributed to the problems in analyzing a complex boundary mentioned in Materials and Methods. We can be confident, however, that the *s* value of 5.55 S (Figure 4d and ref 9) for the solution proportions of four cytochrome c_{550} molecules per cytochrome *c* peroxidase is close to the value for the saturated complex.

In the additional presence of horse cytochrome *c* in proportions of 1:1 with the cytochrome *c* peroxidase (absorbance profile iii of Figure 3), there is a further increase in the sedimentation coefficient of the leading boundary. Using the same method of boundary absorbance analysis outlined above, we can estimate that 63% of the horse cytochrome *c* is bound (assuming that the distribution of cytochrome c_{550} between bound and free remains unaltered). This indicates that the horse cytochrome *c* is binding to a site distinct from the cytochrome c_{550} site.

The $s_{20,w}$ value of this ternary complex boundary is 5.81 S, higher than the value of 5.55 S obtained for a 4:1 solution of cytochrome c_{550} and cytochrome *c* peroxidase (Figure 4d and ref *9*). This effect of the horse cytochrome *c* cannot simply be due to competitive displacement because this cytochrome has a lower molecular weight than the cytochrome c_{550}. The $s_{20,w}$ value of 5.81 S together with an estimate of *D* can be used to derive a molecular weight for a 1:1:1 ternary complex of the three proteins of 107 000. The theoretical molecular weight for such a complex is 129 378. The shortfall will be in part due to the problems associated with analysis of the complex boundary and partly due to the incomplete occupancy of the horse cytochrome *c* site.

Table 1: Thermodynamic Parameters for the Binding of Horse Cytochrome *c* with Cytochrome *c* Peroxidase and with the Binary Complex of Cytochrome *c* Peroxidase and Cytochrome c_{550}

titrant	target	analysis	*N*	K_a ($\times 10^{-5}$ M^{-1})	K_d (μM)	$\Delta H°$ (kJ/mol)
horse cyt *c*	ccp alone	two sets of sites	(1)	10.2 ± 1.8	0.98	19.7 ± 0.7
			(1)	1.6 ± 0.1	6.2	10.3 ± 0.7
	ccp and cyt c_{550}	one set of sites	(1)	0.11 ± 0.005	88	$(37.0 \pm 1.1)^a$
Paracoccus c_{550}	ccp alone	one set of sites	1.0 ± 0.01^b	2.8 ± 0.15	3.6	11.5 ± 0.17
	ccp and horse cyt *c* (1:1)	one set of sites	0.35 ± 0.05	2.2 ± 0.6	4.6	3.6 ± 0.57

[a] This $\Delta H°$ is reported for information only and should not be considered reliable in such weak affinity situations. [b] The goodness of fit is indicated by the standard deviations for the independent variables.

Isothermal Titration Microcalorimetry. (i) Binding of Horse Cytochrome c to a Binary Complex of Cytochrome c_{550} and Cytochrome c Peroxidase. As previously reported (*9*), the binding of horse cytochrome *c* to cytochrome *c* peroxidase is endothermic under these conditions, with an ITC differential binding curve inconsistent with simple 1:1 complexation. The data do, however, fit consistently well to a two-site binding model. The results in Table 1 for the binding of horse cytochrome *c* to cytochrome *c* peroxidase alone (repeated here with the same batch of protein used for the mixed ternary complexation studies) again show a higher-affinity site and a lower-affinity site with binding parameters very similar to those published (*9*) ($K_{d1} = 0.9$ μM, $\Delta H =$ 19.8 kJ/mol; $K_{d2} = 5.9$ μM, $\Delta H = 10.4$ kJ/mol). However, when *Paracoccus* cytochrome c_{550} is also present prior to the titration with horse cytochrome *c*, a much smaller endothermic heat change is seen (part ii of Figure 5A and part ii of Figure 5B). Unambiguous analysis of this ITC curve is not feasible because of the small heat effects and the apparent weak binding, but if we assume $N = 1$, the data are consistent with an apparent K_d of 88 μM (Table 1).

(ii) Binding of Cytochrome c_{550} to a Binary Complex of Horse Cytochrome c and Cytochrome c Peroxidase. The results of Table 1 for the binding of cytochrome c_{550} to cytochrome *c* peroxidase alone show binding parameters very similar to those published (*9*) ($K_d = 3.7$ μM, $\Delta H = 10.4$ kJ/mol). There is an exothermic effect for the dilution of cytochrome c_{550} into buffer alone (part iv of Figure 6A and part iv of Figure 6B). Cytochrome c_{550} is known to be in a monomer−dimer equilibrium under these conditions, and the dilution effect is consistent with a monomerization as the titrant is diluted into the target buffer. That heat change would be expected to decrease as the titration progresses, and the concentration of cytochrome c_{550} in the target solution increases. This effect is apparent from the results of part iv of Figure 6A and part iv of Figure 6B.

Two experiments were performed with target solutions of one horse cytochrome *c* per peroxidase and 3.8 horse cytochrome *c* molecules per peroxidase. On the basis of the K_d values obtained from the binding of horse cytochrome *c* to the peroxidase alone and taking into account the concentration conditions of the experiment, we determine the predicted occupancy of the two sites on the peroxidase for horse cytochrome *c*, shown in Table 2.

When horse cytochrome *c* is present in proportions of 3.8:1 to the peroxidase prior to the titration with cytochrome c_{550}, the observed heat changes (part iii of Figure 6A and part iii of Figure 6B) are not greatly different from those of dilution into buffer alone (part iv of Figure 6A and part iv of Figure 6B). Competitive displacement of the horse cytochrome *c* as the cytochrome c_{550} concentration increases may account for what difference there is [although there is little enthalpy difference in the binding of the two proteins (Table 1)]. In contrast, with horse cytochrome *c* in the proportion of 1:1 with the peroxidase prior to the titration, an endothermic binding can be observed (part ii of Figure 6B). After allowing for the dilution effect into buffer, we can fit the corrected heat change data to 0.35 site with a binding constant of 4.6 μM (Table 1), not too different from the binding constant for binding of cytochrome c_{550} to the peroxidase alone.

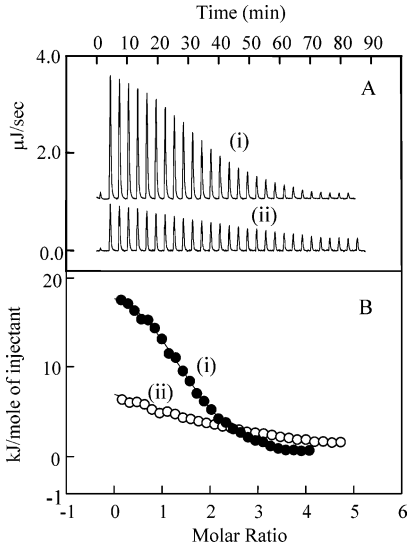

FIGURE 5: Isothermal titration calorimetry of the binding of horse cytochrome *c* to *Paracoccus* cytochrome *c* peroxidase in the presence and absence of cytochrome c_{550}. (A) Horse cytochrome *c*, *Paracoccus* cytochrome c_{550}, and *Paracoccus* cytochrome *c* peroxidase were equilibrated in 10 mM cacodylate (pH 6.0), 10 mM NaCl, and 2 mM $CaCl_2$ by being passed down a Sephadex G25 molecular exclusion column. The microcalorimetry chamber at 25 °C contained degassed cytochrome *c* peroxidase at 23 μM (i) or 20.6 μM (ii), and in the case of part ii, 58 μM cytochrome c_{550} was also present. The syringe contained degassed horse ferricytochrome *c* at 441 μM. Each delivery of titrant was set to take 20 s with 180 s between additions. (B) Heat changes for results obtained in panel A. (i) Titration of *Paracoccus* cytochrome *c* peroxidase with horse cytochrome *c* (●). The best fit of the results is with a two-sets-of-sites model in which *N* is constrained to 1 for each site. Thermodynamic parameters are given in Table 1. (ii) Titration of a solution of *Paracoccus* cytochrome *c* peroxidase and cytochrome c_{550} with horse cytochrome *c* (○). The best fit of the results is with a one-set-of-sites model in which *N* is constrained to 1. Thermodynamic parameters are given in Table 1.

Docking of Cytochromes to Paracoccus Cytochrome c Peroxidase. (i) Binary Complex with Cytochrome c_{550}. For the binary complex formed between cytochrome c_{550} and cytochrome *c* peroxidase, Pettigrew et al. (*8*) found that six of the top 10 ranking docks showed cytochrome c_{550} binding above the E heme of the peroxidase. Five of the six had

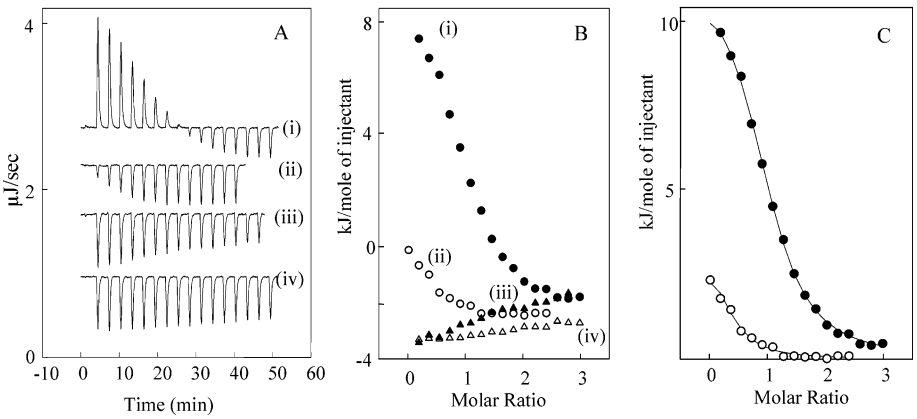

FIGURE 6: Isothermal titration calorimetry of the binding of cytochrome c_{550} to *Paracoccus* cytochrome *c* peroxidase in the presence and absence of horse cytochrome *c*. (A) Horse cytochrome *c*, *Paracoccus* cytochrome c_{550}, and *Paracoccus* cytochrome *c* peroxidase were equilibrated in 10 mM cacodylate (pH 6.0), 10 mM NaCl, and 2 mM $CaCl_2$ by being passed down a Sephadex G25 molecular exclusion column. The microcalorimetry chamber at 25 °C contained degassed cytochrome *c* peroxidase at 23 (i), 21.9 (ii), or 19.2 μM (iii) or buffer only (iv). In the case of part ii, 21.8 μM horse cytochrome *c* was also present, and in the case of part iii, 73.5 μM horse cytochrome *c* was also present. The syringe contained 565 μM degassed cytochrome c_{550}. Each delivery of titrant was set to take 20 s with 180 s between additions. (B) Heat changes for results obtained in panel A. (i) Titration of *Paracoccus* cytochrome *c* peroxidase with cytochrome c_{550} (●). (ii) Titration of a 1:1 solution of *Paracoccus* cytochrome *c* peroxidase and horse cytochrome with *Paracoccus* cytochrome c_{550} (○). (iii) Titration of a 1:3.8 solution of *Paracoccus* cytochrome *c* peroxidase and horse cytochrome with *Paracoccus* cytochrome c_{550} (▲). (iv) Titration of *Paracoccus* cytochrome c_{550} into buffer alone (△). (C) Best fit of results obtained for parts i and ii of panel B after correction for the dilution effect and using a single-set-of-sites model. The thermodynamic parameters are given in Table 1.

Table 2: Occupancy of the Two Sites on Cytochrome *c* Peroxidase that Bind Horse Cytochrome *c*

		occupancy (%)[a]	
	K_d (μM)	1:1 horse cyt *c*:ccp	3.8:1 horse cyt *c*:ccp
site 1	0.98	67	98
site 2	6.2	25	87

[a] Under the conditions of the isothermal microcalorimetry experiment.

very similar orientations (we will call these type I), and this is represented by the top-ranking solution shown in Figure 8G. In one of the six, the orientation was rotated 180° around the region of contact with the peroxidase (Figure 8A). We will call this type II. These orientations can be distinguished in Figure 8 by examining the position of the C-terminus (indicated by an arrow) and a loop of chain (indicated with an asterisk). These docks were done with an early version of BiGGER which has subsequently been refined.

In the more recent version of BiGGER, docking can be performed either by considering the molecules as rigid bodies (hard docking) or by allowing for side chain mobility and uncertainty in surface structure (soft docking). In the case of cytochrome c_{550}, it is the soft docking mode that produces very clear-cut results, comparable to those already published using the original version of BiGGER. Again six of the top 10 ranking solutions showed cytochrome c_{550} bound above the E heme site (Figure 7A). [Notice that this dock involves the peroxidase monomer and four docking solutions are excluded from the top 10 list because the cytochrome c_{550} is bound at the dimer interface, a structural impossibility in solution where the active enzyme is dimeric (*17*). These interface solutions are on the right side of Figure 7B.] Again there are two types of solution which match the types I and II seen with the original BiGGER program, and these two types are related by a rotation of 180° around the region of contact with the peroxidase. This time only two of the six are in the type I orientation (Figure 8H,I), and the other four are in the type II orientation (Figure 8B−E). It is noticeable that the type I orientation seems to allow a slightly closer approach of the iron of the cytochrome c_{550} to the heme iron of the E heme of the cytochrome *c* peroxidase (distances in the figure are in angstroms).

Within the top 500 docking solutions, there is a large cluster that is situated above the E heme (Figure 7A). In the vast majority of these and in all of the group in the top 10 ranked solutions, the orientation of the heme of the cytochrome c_{550} is toward the surface of the cytochrome *c* peroxidase, thus minimizing the iron−iron distance and achieving a close approach of the edges of the cytochrome heme and the peroxidase E heme. Only a few solutions have the opposite orientation with the heme of the cytochrome c_{550} turned outward. These outliers are visible in Figure 7B (indicated by the arrow). In the hard docking mode, there is a much smaller cluster of solutions situated above the E heme (Figure 7C), and of these, only three are ranked in the top 10.

(ii) Binary Complex with Horse Cytochrome c. For the binary complex formed between horse cytochrome *c* and cytochrome *c* peroxidase, six of the top 10 ranking docks showed horse cytochrome *c* binding between the two hemes of the peroxidase (*8*). Using the refined version of BiGGER, it is the hard docking mode that, in this case, produces a pattern (Figure 9A) similar to the published pattern in ref *8*. Again six of the top 10 ranking solutions were located between the two peroxidase hemes (the between hemes cluster of Figure 9A) rather than at the E heme location that is observed with the cytochrome c_{550}. [Notice that in the views presented in Figure 9, the hemes of these docking solutions appear to be closer to the P heme of the peroxidase rather than between the two hemes. However, the P heme is buried and the iron−iron distances from the cytochrome to the two heme groups are very similar (see, for example, Figure 10A,B).] The top-ranking solution from the original published dock is shown in Figure 10A, and a top-ranking solution from the current hard docking is shown in Figure 10B.

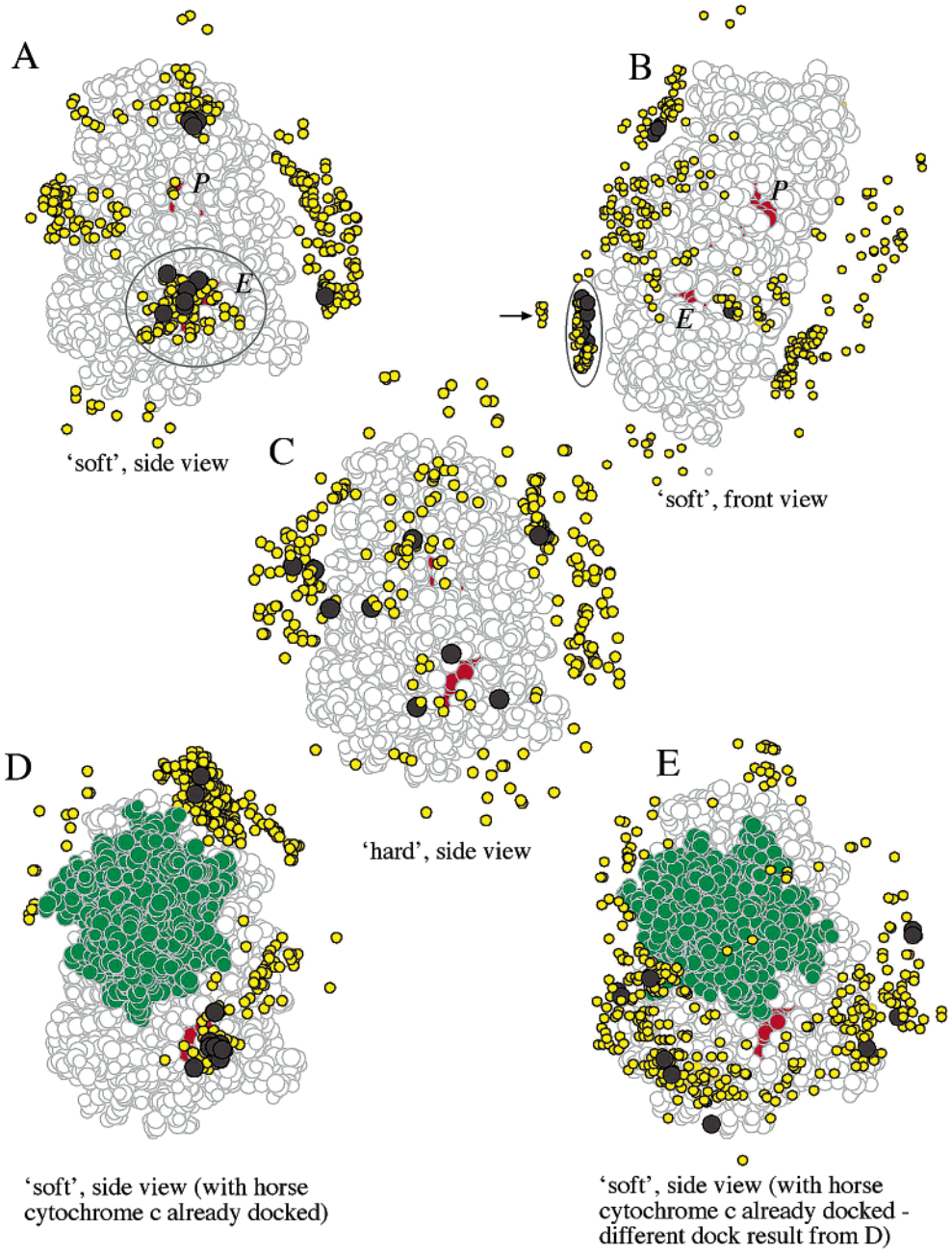

FIGURE 7: Docking of cytochrome c_{550} to cytochrome *c* peroxidase of *P. denitrificans*. The side of the peroxidase monomer at which the E heme edge is exposed (A and C−E) or the front of the monomer (B) is shown as faint gray empty cicles with the P and E hemes in red and partially visible. The two views are related by a 90° rotation about a vertical axis. The E heme is the more exposed and is in the lower half of the peroxidase structure; the P heme is mostly buried, and portions of it can be seen in the upper half of the peroxidase molecule unless concealed by docking clusters. In panels D and E, the docked horse cytochrome *c* molecule is shown in green. In all simulations, the 500 most favorable solutions are shown as small yellow circles marking the heme iron of the probe protein, cytochrome c_{550}. The top 10 docking solutions are shown as larger circles filled with dark gray. The E heme cluster is circled in panels A and B. (A) Side view of the soft docking of cytochrome c_{550} to the peroxidase monomer. (B) Front view of the soft docking of cytochrome c_{550} to the peroxidase monomer. The arrow denotes outlying heme iron positions which reflect cytochrome c_{550} orientations facing away from the molecular surface of the peroxidase. The solutions at the right side are situated at what would be the dimer interface of the peroxidase and can be ignored. (C) Side view of the hard docking of cytochrome c_{550} to the peroxidase monomer. (D) Side view of the soft docking of cytochrome c_{550} to a binary complex of the cytochrome *c* peroxidase and horse cytochrome *c*. The top-ranking solution of this dock is shown in Figure 8F. The binary complex that was used was the third-ranking solution (Figure 10B) of the hard dock shown in Figure 9A. (E) Side view of the hard docking of cytochrome c_{550} to a binary complex of the cytochrome *c* peroxidase and horse cytochrome *c*. The binary complex that was used was the top-ranking solution of the published dock using the original version of BiGGER and is shown in Figure 10A.

The results for the soft docking mode are quite different (Figure 9B,C). Although the between hemes location is still populated, a large proportion of the top 500 solutions and six of the top 10 solutions are located above the E heme. However, the problem with these solutions can be seen by comparing Figure 9C with Figure 7B. Both show strong clusters above the E heme, but in the case of this soft dock for horse cytochrome *c*, almost all the heme iron locations for the horse cytochrome *c* are further from the molecular surface and reflect heme orientations turned outward. An example of this is shown in Figure 10C.

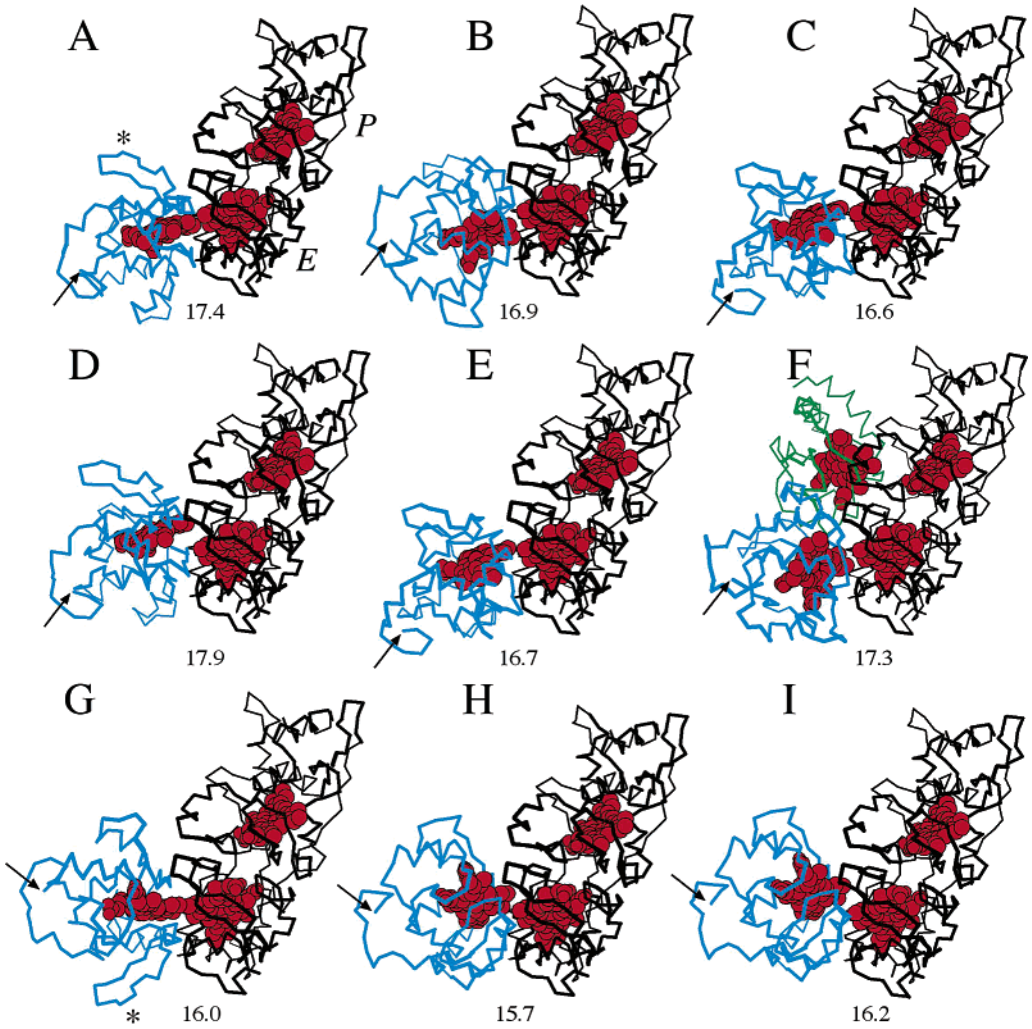

FIGURE 8: Top-ranking solutions for cytochrome c_{550} docking to cytochrome *c* peroxidase of *P. denitrificans*. The backbone of the cytochrome *c* peroxidase monomer is shown from the front enclosing the E heme (lower) and the P heme (upper) filled in red. This view is equivalent to that of Figure 7B. The probe cytochrome c_{550} heme is shown in red enclosed by a blue polypeptide backbone. The iron−iron distances between the cytochrome c_{550} heme and the E heme iron of the cytochrome *c* peroxidase are indicated (angstroms). Panels A−F are described as type II solutions as explained in the accompanying text. Panels G−I are type I solutions. These cytochrome c_{550} orientations are related by a rotation of approximately 180°, and this rotational relationship between type I and type II solutions is indicated by the arrows pointing to the C-terminus of the cytochrome c_{550} backbone and is also evident in most cases in the position of the polypeptide loop marked with an asterisk which is below and above the heme group, respectively. (A) Top-ranking type II solution from the top 10 docks obtained using the original version of BiGGER (*8*). (B−E) Four type II solutions from the six top-ranking solutions above the E heme in the soft docking shown in panels A and B of Figure 7. (F) Top-ranking solution from the soft dock of cytochrome c_{550} to a binary complex of horse cytochrome *c* (shown in green) and cytochrome *c* peroxidase. The overall docking results are shown in Figure 7D, and the binary complex that was used is shown in Figure 10B and was the third-ranking solution of the hard dock shown in Figure 9A. (G) Top-ranking type I solution from the top 10 docks obtained using the original version of BiGGER (*8*). (H and I) Two type I solutions from the six top-ranking solutions above the E heme in the soft docking shown in panels A and B of Figure 7.

(iii) Ternary Complexes. If the top type I orientation of the cytochrome c_{550} against the peroxidase from ref *1* (Figure 8G) is used as a target for the ternary docking of horse cytochrome *c*, nine of the top 10 ternary complexes have the horse cytochrome *c* positioned between the two peroxidase heme groups (Figure 9D). An example of one of these high-ranking ternary docks in which the orientation of the horse cytochrome *c* is similar to the binary dock of Figure 10B is shown in Figure 10D. If the top type II orientation of the cytochrome c_{550} against the peroxidase (Figure 8B) is used as a target, there is again a good cluster of docks situated between hemes with eight of the top 10 docks in this position (Figure 9F). An example from this cluster that again resembles the orientation of horse cytochrome *c* in Figure 10B is shown in Figure 10E. (The horse cytochrome *c* in panel D is rotated in the plane of the page relative to panel B so that the N-terminal helix is closer to the peroxidase. This is also the case in panel E but with an additional rotation of the "bottom" of the molecule given by the position of the protruding propionate groups of the heme.) It therefore seems that either the type I or the type II orientation observed in the binary docks with cytochrome c_{550} and cytochrome *c* peroxidase is permissive as a binding target for horse cytochrome *c*. However, interestingly, the type I relative shown in Figure 8H in which the cytochrome c_{550} heme is rotated about 30° relative to its position in Figure 8G shows a quite different docking pattern with horse cytochrome *c* (Figure 9E) because the cytochrome c_{550} interferes with the between hemes surface.

The critical importance of the orientation of the bound cytochrome in the target for ternary docking is also seen with the docking of cytochrome c_{550} to a horse cytochrome

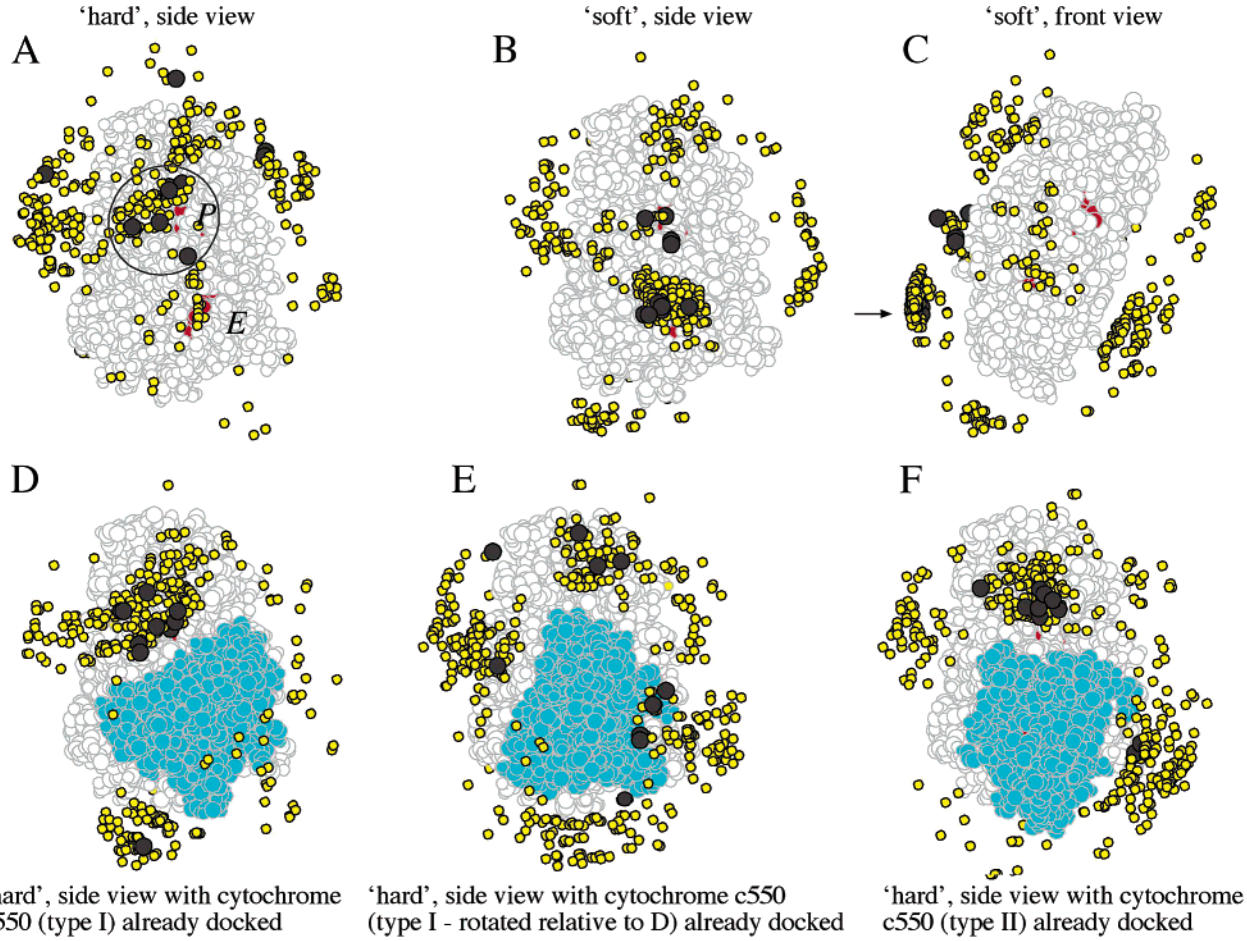

FIGURE 9: Docking of horse cytochrome *c* to cytochrome *c* peroxidase of *P. denitrificans.* The side of the peroxidase monomer at which the E heme edge is exposed (A, B, and D−F) or the front of the monomer (C) is shown in faint gray empty circles with the P and E hemes in red and partially visible. The two views are related by a 90° rotation about a vertical axis. The E heme is more exposed and is in the lower half of the peroxidase structure; the P heme is mostly buried, and portions of it can be seen in the upper half of the peroxidase molecule. In panels D−F, the docked cytochrome c_{550} molecule is shown in cyan. In all simulations, the 500 most favorable solutions are shown as small yellow circles marking the heme iron of the probe protein, horse cytochrome *c*. The top 10 docking solutions are shown as larger circles filled with gray. The between hemes cluster is circled in panel A. (A) Side view of the hard docking of horse cytochrome *c* to the peroxidase monomer. (B) Side view of the soft docking of horse cytochrome *c* to the peroxidase monomer. (C) Front view of the soft docking of horse cytochrome *c* to the peroxidase monomer. The arrow indicates outlying heme iron positions which reflect horse cytochrome *c* orientations facing away from the molecular surface of the peroxidase. The solutions at the right side are situated at what would be the dimer interface of the peroxidase and can be ignored. (D) Side view of the hard docking of horse cytochrome *c* to a binary complex of the cytochrome *c* peroxidase and cytochrome c_{550}. An example of a top 10 ranking solution of this dock is shown in Figure 10D. The binary complex that was used was the highest-ranking type I dock from the top 10 docks obtained using the original version of BiGGER (*8*). This binary complex is shown in Figure 8G. (E) Side view of the hard docking of horse cytochrome *c* to a binary complex of the cytochrome *c* peroxidase and cytochrome c_{550}. The binary complex that was used was a type I dock with an orientation slightly different from that in Figure 8G taken from the top 10 solutions of Figure 7A. This binary complex is shown in Figure 8H. (F) Side view of the hard docking of horse cytochrome *c* to a binary complex of the cytochrome *c* peroxidase and cytochrome c_{550}. An example of a top 10 ranking solution of this dock is shown in Figure 10E. The binary complex that was used was the highest-ranking type II dock taken from the top 10 solutions of Figure 7A. This binary complex is shown in Figure 8B.

c−cytochrome *c* peroxidase target. If the binary solution shown in Figure 10A is used a target, there are no high-ranking solutions found that are positioned above the E heme (Figure 7E). However, if the binary solution shown in Figure 10B is used as a target, then cytochrome c_{550} can bind above the E heme (Figure 7D) in eight of the top 10 ranking solutions. The top-ranking example of this which has an orientation of the cytochrome c_{550} close to type II is shown in Figure 8F.

(iv) The Top 10 Ranking Solutions. In Figures 7 and 9, we have emphasized the top 10 solutions in dark gray and with larger circles. To some extent, how many of the top-ranking solutions are emphasized is arbitrary. However, Figure 11 shows (at least for the case of the soft docking of cytochrome c_{550} to the peroxidase) that there is some justification for emphasizing the top 10 solutions as they are characterized by a step change in the global score. Figure 11 also shows the clustering of top-ranking solutions (boxed) with close approach of the cytochrome heme group to the E heme of the peroxidase.

DISCUSSION

Ternary Complex of Horse Cytochrome c, Cytochrome c_{550}, and Cytochrome c Peroxidase. We are interested in using a variety of methods to study the transient complexes formed by electron transfer proteins, partly because such complexes are harder to demonstrate than tight complexes and partly because the different methods cast different lights

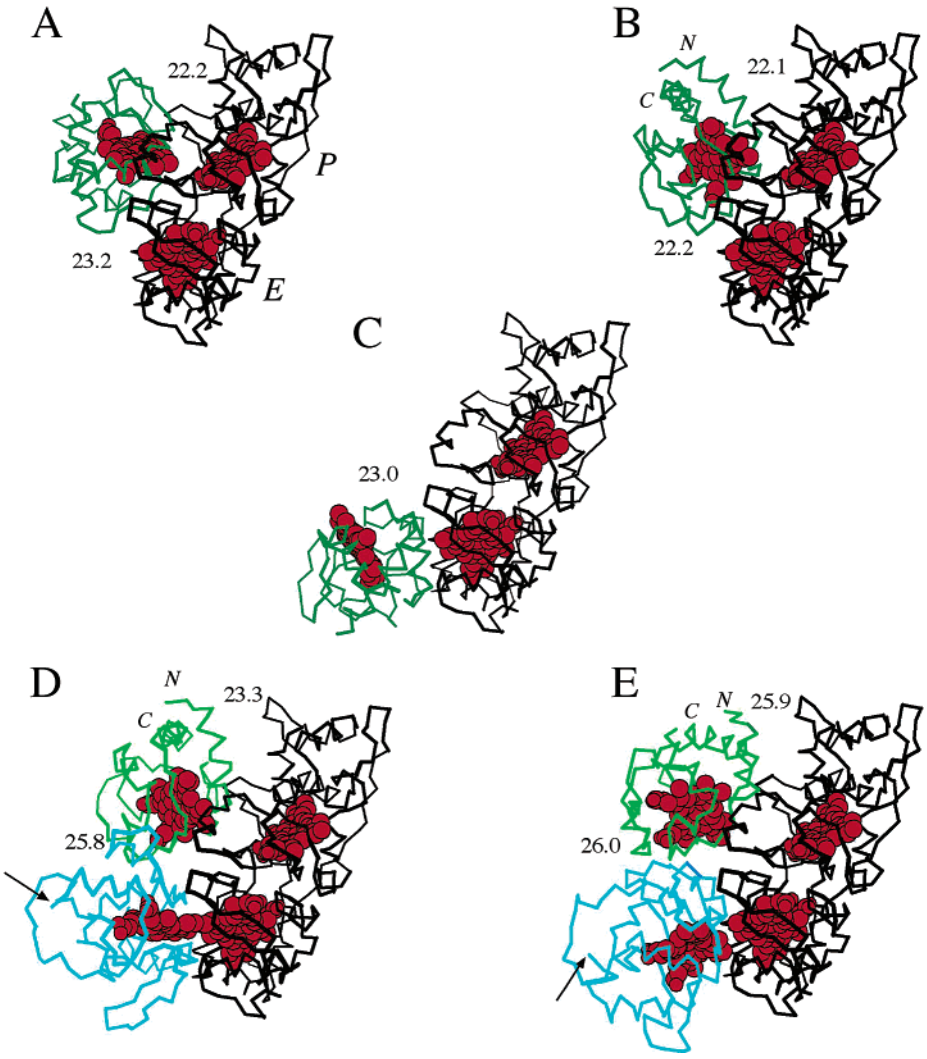

FIGURE 10: Top-ranking solutions for horse cytochrome *c* docking to cytochrome *c* peroxidase of *P. denitrificans*. The backbone of the cytochrome *c* peroxidase monomer is shown from the front enclosing the E heme (lower) and the P heme (upper) in red. The probe horse cytochrome *c* heme is shown in red enclosed by a green polypeptide backbone. The iron−iron distances between the horse cytochrome *c* heme and the two heme irons of the cytochrome *c* peroxidase are indicated (angstroms). The upper number in each case is the horse cytochrome *c* heme iron−P heme iron distance; the lower number is the horse cytochrome *c* heme iron−E heme iron distance. In some of the frames, the N- and C-terminal α-helices of the horse cytochrome *c* molecule are indicated. (A) Top-ranking solution from the top 10 docks obtained using the original version of BiGGER (*8*). This was used as the target for the ternary docking shown in Figure 7E. (B) Example of a top 10 solution from the hard docking shown in Figure 9A. This was used as the target for the ternary docking shown in Figure 7D. (C) Top-ranking solution from the soft dock of horse cytochrome *c* to cytochrome *c* peroxidase shown in panels B and C of Figure 9. (D) One of the top 10 ranking solutions of the ternary dock (Figure 9D) of horse cytochrome *c* to the type I binary target shown in Figure 8G. (E) One of the top 10 ranking solutions of the ternary dock (Figure 9F) of horse cytochrome *c* to the type II binary target shown in Figure 8B.

on the nature of electron transfer complexes. Of the methods used here, we have strong evidence from ^{1}H NMR spectroscopy and sedimentation velocity centrifugation that a ternary complex can be formed between cytochrome *c* peroxidase, horse cytochrome *c*, and cytochrome c_{550}.

In the case of ^{1}H NMR spectroscopy, with the three proteins present in equimolar proportions, it is clear that the cytochrome c_{550} is fully bound and horse cytochrome *c* is ~75% bound. The essentially complete binding of the cytochrome c_{550} under these conditions is reasonably consistent with a 91% occupancy calculated from the K_d value (4.3 μM) obtained by microcalorimetry under the higher-ionic strength conditions of this NMR experiment (*9*). Horse cytochrome *c* alone with the cytochrome *c* peroxidase would be expected to partition between the higher- and lower-affinity binding sites according to the K_d values of 11.6 and 25.9 μM obtained by microcalorimetry under comparable conditions (*9*). Using these values, site occupancies for horse cytochrome *c* under the conditions in the NMR experiment are predicted to be 59 and 39%, respectively. We propose that, when all three proteins are present, cytochrome c_{550} preferentially occupies the lower-affinity binding site for horse cytochrome *c* and some displacement of horse cytochrome *c* is therefore observed. But the important point is that a ternary complex is detected in which the cytochrome c_{550} site is fully occupied and the remaining horse cytochrome *c* site is mostly occupied.

The existence of such a complex is confirmed by sedimentation velocity ultracentrifugation. Analysis of the leading and trailing boundaries suggests that the cytochrome c_{550} site is almost fully occupied under the conditions of absorbance profile ii of Figure 3, and yet when the smaller

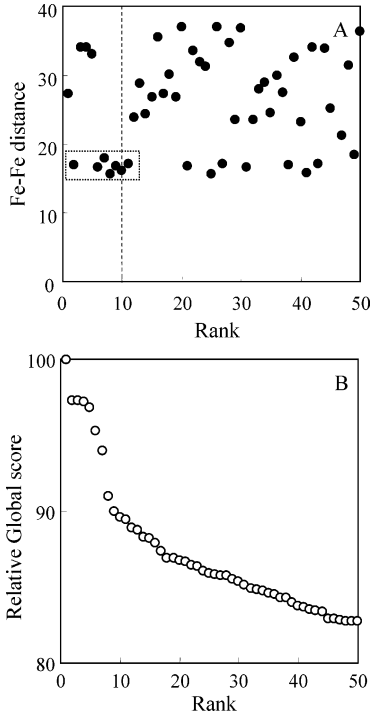

FIGURE 11: Analysis of the high-ranking solutions from the soft docking simulation of cytochrome c_{550} to the peroxidase monomer (Figure 7A,B). (A) The distance between the iron of the cytochrome c_{550} and the iron of the electron-transferring heme is shown for the top 50 ranked solutions. The boxed solutions are those that have both a high global score and a close approach of the two heme groups. (B) The global score is shown for the top 50 solutions expressed relative to that of the top solution (100%). This is a number derived from the combination of the interaction criteria described in Materials and Methods.

horse cytochrome *c* is also present, a further increase in the *s* value is observed (absorbance profile iii of Figure 3). This can only be explained by horse cytochrome *c* occupying a distinct vacant site on the cytochrome *c* peroxidase surface. Under these conditions, higher-affinity horse cytochrome *c* binding is associated with a K_d value of 0.98 μM obtained by microcalorimetry (*9*) (Table 1). Using this value, the occupancy of the higher-affinity horse cytochrome *c* site in the ultracentrifugation experiment is predicted to be 76% which is comparable to the value of 63% obtained by calculation from the boundary absorbances. This level of occupancy partially explains the shortfall in the estimate of M_r for the ternary complex.

Thus, on the basis of the results of NMR and ultracentrifugation and with reference to the binding model proposed in ref *8*, we propose that a ternary complex can be formed by cytochrome c_{550} binding to a single site at the E heme and horse cytochrome *c* binding to a second site between the two hemes. Horse cytochrome *c* alone can form a complex containing two molecules of horse cytochrome *c* and one of the peroxidase monomer (*9*). We propose that this second site for horse cytochrome *c* is the E heme site at which cytochrome c_{550} also binds.

Molecular Docking Simulations. (i) Binary Docks. We should not believe the molecular docking simulations provide definitive independent answers; we should view them in the context of the results obtained by ^{1}H NMR spectroscopy and ultracentrifugation which demonstrate that ternary complexes can exist and can be interpreted in terms of a peroxidase surface with two distinct binding sites. As shown previously (*8*) and confirmed here in the new hard docking simulation of Figure 9A, the favored binding surface for the binding of horse cytochrome *c* is between hemes, and we propose that this represents the higher-affinity site observed by microcalorimetry. Previously published work had shown that the favored binding surface for the cytochrome c_{550} is at the exposed edge of the E heme (*8*), and this is confirmed by the new soft docking experiment depicted in panels A and B of Figure 7. We propose that this represents the single site for cytochrome c_{550} observed by microcalorimetry. According to the molecular docking simulation, a cytochrome c_{550} occupying that site can adopt one of two main orientations (called type I and type II) which are related by a rotation of 180° about a perpendicular to the molecular surface of the peroxidase (Figure 8). These may represent real alternative binding modes.

It is the different geometric relationships between the cytochrome c_{550} and the horse cytochrome *c* bound at these two sites and the E heme of the cytochrome *c* peroxidase that give rise to the different patterns of chemical shift perturbation observed in the downfield-shifted methyl resonances of this heme (*8*). It was this observation that originally gave rise to the proposal that two distinct binding sites on the molecular surface were present.

We have chosen to emphasize the results of the hard docking for horse cytochrome *c* and the soft docking for the cytochrome c_{550}. In the case of the horse cytochrome *c*, the same sort of high-ranking between hemes solution is retained in both the hard and soft docking (Figure 9A,B), but the soft docking algorithm also retains a very large number of solutions at the E heme position, none of which have a heme orientation compatible with electron transfer at the exposed heme edge (Figure 9C). Thus, in this case, hard docking retains relevant solutions without the need for the more permissive procedure of soft docking (which simply introduces a higher level of noise). In the case of the cytochrome c_{550}, the soft docking also retains a very large number of solutions at the E heme (Figure 7A), but in this case, a high proportion of these, including high-ranking solutions, have heme orientations compatible with electron transfer (Figure 7B). The hemes of the donor cytochromes and the E heme of the peroxidase have edges exposed at the protein surface at which we assume electron transfer occurs. A productive complex will therefore have a juxtaposition of these heme edges as shown in Figure 8, while Figure 10C, for example, would not be expected to be productive. Such solutions are retained in the hard docking (Figure 7C) but to a much smaller degree. In this case, we would argue that the more permissive docking is required for a good proportion of these relevant solutions to be retained. It may be that the different docking requirements of the two cytochromes reflect the real difference in their relationship with the peroxidase. One can argue that the physiological donor, cytochrome c_{550}, has evolved to form specific salt bridges with the peroxidase surface and these can only be accommodated in a docking procedure if flexibility in the position of side chains is permitted. On the other hand, the nonphysiological donor, horse cytochrome *c*, may interact on a more global electrostatic basis, and this can be accommodated by the rigid body approach of the hard docking algorithm.

(ii) Ternary Docks. It is clear from the ternary docking experiments that a binary complex of cytochrome c_{550} and cytochrome *c* peroxidase can physically accommodate a horse cytochrome *c* in the between hemes site (Figure 9D,F) and with an orientation (Figure 10D) that resembles high-ranking solutions of the binary docking of horse cytochrome *c* with the enzyme (Figure 10B). It is also clear from the ternary docking simulations that a binary complex of horse cytochrome *c* and cytochrome *c* peroxidase can physically accommodate a cytochrome c_{550} in the E heme site (Figure 7D) and with an orientation (Figure 8F) that resembles high-ranking solutions of the binary docking of cytochrome c_{550} with the enzyme (Figure 8A−E).

We should emphasize that there are uncertainties in the process of ternary docking in addition to those encountered in binary docking simulations. One is that the training of the program was done using binary complexes. A second is that it is likely that an incoming ternary partner would influence the binding of the binary partner. And finally and probably most importantly, we can see from the results in panel E of Figures 7 and 9 that the choice of the particular binary complex as a target can have a dramatic effect on the results of the ternary docking experiment.

Isothermal Microcalorimetry. The most difficult data to interpret are those of the microcalorimetry experiments. Previous ITC experiments (*9*), also reproduced here, have shown that horse cytochrome *c* can bind simultaneously at two sites on cytochrome *c* peroxidase, albeit with different affinities, thus demonstrating the existence of a ternary complex comprising one cytochrome *c* peroxidase with two horse cytochrome *c* molecules. The question we wish to address here is whether it is feasible to form a *mixed* ternary complex comprising cytochrome *c* peroxidase, horse cytochrome *c*, and cytochrome c_{550}. Unfortunately, the ITC data do not give a clear answer. This is partly because of the complicated mixture of heat changes that will be occurring due to probable competition effects and partly because one cannot design an experiment in which one horse cytochrome *c* site is occupied while the other is empty.

In the experiment whose results are depicted in part ii of Figure 5, in which horse cytochrome *c* is added to a solution containing cytochrome c_{550} and cytochrome *c* peroxidase, there are two possible origins for the observed heat changes. They may represent horse cytochrome *c* binding to one of the two sites seen in the absence of cytochrome c_{550} but with a weakened affinity (K_d = 88 μM) due to the presence of the latter. The second alternative is that there is competition between the horse cytochrome *c* and the cytochrome c_{550}. These possibilities are now discussed.

If we assume a K_d value of 3.6 μM for the binding of cytochrome c_{550} (Table 1), we can calculate that, under the conditions of the experiment whose results are depicted in part ii of Figure 5, the single cytochrome c_{550} site is 92% occupied. If horse cytochrome *c* binding was in competition with that of cytochrome c_{550}, the binding constant would be predicted to be altered by a factor of $1 + [I]/K_I$, where [I] is the concentration of cytochrome c_{550} and K_I is the dissociation constant for cytochrome c_{550}. Thus, competition at the high-affinity horse cytochrome *c* site would be expected to yield an apparent binding constant of 16.8 μM and competition at the low-affinity site a binding constant of 106 μM. Since the actual fit of part ii of Figure 5B is 88 μM, competition at the lower-affinity site seems to be, at first sight, a feasible scenario. However, we must remember that the overall heat change observed would be a composite of the change due to the binding of horse cytochrome *c* and the change due to the dissociation of cytochrome c_{550}. Since the binding of both is endothermic with similar ΔH values (10.3 and 11.5 kJ/mol, respectively), the binding of one, coupled to the dissociation of the other, should be essentially athermic. Thus, the more likely of the two possibilities would appear to be that the horse cytochrome *c* is binding to a second site on the cytochrome c_{550}−cytochrome *c* peroxidase binary complex. However, one surprising feature of this binding is the weakened affinity, something not suggested by the other methodologies employed here. A second surprising feature is the much higher $\Delta H°$ value (Table 1) (although this may be a consequence of the long extrapolation required for fitting the weaker binding).

Experiments were also performed in the reciprocal mode with horse cytochrome *c* already bound to the cytochrome *c* peroxidase and cytochrome c_{550} as the titrant. These experiments are more difficult to design satisfactorily because, unlike the binding of cytochrome c_{550} to the enzyme, conditions cannot be created whereby one of the two horse cytochrome *c* sites is mostly filled while the other is mostly empty. With a horse cytochrome to peroxidase ratio of 3.8:1 prior to titration with cytochrome c_{550}, there is no sign of the endothermic changes associated with binding of cytochrome c_{550}, and this is consistent with the high occupancy of both its sites by horse cytochrome *c* under these conditions (Table 2). This implies the cytochrome c_{550} shares a site with horse cytochrome *c*. In contrast, with horse cytochrome *c* in the proportion of 1:1 with the peroxidase prior to the titration, an endothermic binding to 0.35 site with a binding constant of 4.6 μM is observed, not too different from the K_d for binding of cytochrome c_{550} to the peroxidase alone. If cytochrome c_{550} is binding to the lower-affinity horse cytochrome *c* site, at least part of the heat change that is observed may, in fact, be due to the horse cytochrome *c* that is displaced from this weaker binding site, and then binding endothermically to the partially occupied higher-affinity site. It may be that this complexity in the heat changes that are occurring leads to the observed anomalous molar ratio.

Thus, the evidence for formation of a ternary complex from these two experiments alone is not strong. Only with the input of the other methodologies can we be sure that a ternary complex is present.

Binding Sites and Electron Transfer Sites. What this work does not address is whether the ternary complexes that are formed are active in electron transport. When the mixed-valence enzyme is oxidized by hydrogen peroxide, two electrons are lost, and these must be replaced by electron transfer. The presence of distinct binding sites for horse cytochrome *c* and cytochrome c_{550} raises the possibility that the required electrons might be delivered synchronously. In the case of cytochrome c_{550}, docking at the E heme yields complexes in which the distance between the iron atoms is around 16 Å and the heme edges of the two proteins are virtually touching (Figure 8), an arrangement that is certainly compatible with the expected geometric constraints of electron transfer. It is less certain whether the docking of horse cytochrome *c* between the two heme groups of the peroxidase also represents an electron-transferring complex

since the iron−iron distances are somewhat larger (Figure 10). Pettigrew et al. (*9*) addressed the question of whether the high-affinity binding of horse cytochrome *c* was productive in electron transfer and noted that the looser binding to the site as the ionic strength was increased was associated with a marked increase in steady state kinetic activity. They concluded that the higher-affinity binding was, in fact, nonproductive and that only when that attachment was loosened could the horse cytochrome *c* migrate to the true electron transfer site. If we relate these conclusions to the docking simulations, we can propose that the between hemes site is the high-affinity site for horse cytochrome *c* and is not an electron transfer site, while the E heme site is the low-affinity site and is functional in electron transfer. Thus, the possibility of synchronous delivery of two electrons appears to be ruled out.

Of course, horse cytochrome *c* is a nonphysiological redox partner for *Paracoccus* cytochrome *c* peroxidase, but we are working on the assumption that it may be probing a real capture surface on the enzyme. The capture of an "active and waiting" cytochrome *c* on the molecular surface of the cytochrome *c* peroxidase represents a potential mechanism for physiological rate enhancement. Such a mechanism whereby a second cytochrome induces a substrate-assisted dissociation of the first is proposed in the case of the yeast cytochrome *c* peroxidase (*5*). But if that is the case, then it clearly cannot involve the nonphysiological horse cytochrome *c*. Nor can it involve two molecules of the cytochrome c_{550} since there is no indication from any of the methods used that two cytochrome c_{550} molecules could be accommodated on the enzyme at the same time. However, *P. denitrificans* does contain pseudoazurin, a second redox protein that is active as an electron donor to cytochrome *c* peroxidase (S. R. Pauleta, unpublished observations). It is possible that a physiological ternary complex might contain both cytochrome c_{550} and pseudoazurin with implications for the mechanism and rate of electron transfer. Current work is addressing this question. The ready replaceability of the copper in pseudoazurin with non-redox active metals such as cadmium opens up the possibility of studying the influence of the binding of one redox partner on the electron transfer activity of the other. This is difficult with horse cytochrome *c* and cytochrome c_{550} because of the possibility of electron transfer between the two as well as with the peroxidase itself.

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BI034829C