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Amicyanin Transfers Electrons from Methylamine Dehydrogenase to Cytochrome *c*-551i via a Ping-Pong Mechanism, not a Ternary Complex

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Abstract: The first crystal structure of a ternary redox protein complex was comprised of the enzyme methylamine dehydrogenase (MADH) and two electron transfer proteins, amicyanin and cytochrome *c*-551i from *Paracoccus denitrificans* [Chen et al. *Science* **1994**, *264*, 86−90]. The arrangement of the proteins suggested possible electron transfer from the active site of MADH via the amicyanin copper ion to the cytochrome heme iron, although the distance between the metals is large. We studied the interactions between these proteins in solution. A titration followed by NMR spectroscopy shows that amicyanin binds cytochrome *c*-551i. The interface comprises the hydrophobic and positive patches of amicyanin, not the binding site observed in the ternary complex. NMR experiments further show that amicyanin binds tightly to MADH with an interface that matches the one observed in the crystal structure and that mostly overlaps with the binding site for cytochrome *c*-551i. Upon addition of cytochrome *c*-551i, no changes in the NMR spectrum of MADH-bound amicyanin are observed, suggesting that a possible interaction of the cytochrome with the binary complex must be very weak, with a dissociation constant higher than 2 mM. Reconstitution of the entire redox chain in vitro demonstrates that amicyanin can react rapidly with cytochrome *c*-551i, but that association of amicyanin with MADH inhibits this reaction. It is concluded that electron transfer from MADH to cytochrome *c*-551i does not involve a ternary complex but occurs via a ping-pong mechanism in which amicyanin uses the same interface for the reactions with MADH and cytochrome *c*-551i.

Introduction

Intermolecular electron transfer is a fundamental step in respiration. Small soluble redox proteins mediate electron transfer from a substrate-reduced donor to a terminal membrane-bound acceptor. Transient interactions between partners, recognizing each other through specific surfaces, are necessary to bring into proximity and properly orient their redox centers so as to ensure fast electron transfer. A rapid dissociation of the components of the transient complex is crucial to enable high turnover.

Structural studies have become an important tool to gain insight into this process. In particular, several crystal structures of specifically associating redox proteins are available.[1−7] Of specific interest to us is the ternary complex comprising the tryptophan tryptophylquinone (TTQ)-containing methylamine dehydrogenase (MADH), the type 1 copper protein amicyanin, and the cytochrome *c*-551i, three components of a periplasmic respiratory chain of *Paracoccus denitrificans*, the structure of which has been determined to high resolution.[6]

MADH catalyzes the oxidation of methylamine to formaldehyde with formation of the aminoquinol TTQ and the subsequent release of ammonia. The genes coding for MADH and its electron acceptor amicyanin are coordinately expressed and induced when *P. denitrificans* is grown on methylamine as the sole carbon source.[8−10] Amicyanin is required for rapid transfer of electrons from MADH to the *c*-type cytochromes involved in the respiratory chain, as shown by the fact that its inactivation by gene replacement results in a complete loss of the ability to grow on methylamine.[10] MADH and amicyanin

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(1) Pelletier, H.; Kraut, J. *Science* **1992**, *258*, 1748–55.
(2) Kurisu, G.; Kusunoki, M.; Katoh, E.; Yamazaki, T.; Teshima, K.; Onda, Y.; Kimata-Ariga, Y.; Hase, T. *Nat. Struct. Biol.* **2001**, *8*, 117–21.
(3) Leys, D.; Basran, J.; Talfournier, F.; Sutcliffe, M. J.; Scrutton, N. S. *Nat. Struct. Biol.* **2003**, *10*, 219–25.
(4) Dai, S.; Friemann, R.; Glauser, D. A.; Bourquin, F.; Manieri, W.; Schurmann, P.; Eklund, H. *Nature* **2007**, *448*, 92–6.
(5) Chen, L.; Durley, R.; Poliks, B. J.; Hamada, K.; Chen, Z.; Mathews, F. S.; Davidson, V. L.; Satow, Y.; Huizinga, E.; Vellieux, F. M.; Hol, W. G. J. *Biochemistry* **1992**, *31*, 4959–64.
(6) Chen, L.; Durley, R. C.; Mathews, F. S.; Davidson, V. L. *Science* **1994**, *264*, 86–90.
(7) Lange, C.; Hunte, C. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 2800–5.
(8) Husain, M.; Davidson, V. L. *J. Biol. Chem.* **1985**, *260*, 14626–9.
(9) Husain, M.; Davidson, V. L. *J. Bacteriol.* **1987**, *169*, 1712–7.
(10) van Spanning, R. J.; Wansell, C. W.; Reijnders, W. N.; Oltmann, L. F.; Stouthamer, A. H. *FEBS Lett.* **1990**, *275*, 217–20.

have been shown to form a binary complex in solution[11] with a dissociation constant of 4.5 μM under low ionic strength conditions,[12] and its crystal structure has been solved to 2.5 Å resolution.[5] MADH is a heterotetramer composed of two heavy (47 kDa) and two light (15 kDa) chains, with each small subunit containing one TTQ, a redox cofactor originating from post-translational cross-linking of two tryptophan side chains and modification of one of them to contain an orthoquinone function.[13] Amicyanin (11.5 kDa) mainly interacts with the light subunit of MADH via the hydrophobic patch that surrounds the copper binding site. In the crystal, the copper atom is positioned at 16.8 Å from the reactive oxygen of the TTQ cofactor and 9.3 Å from its closest atom. The electrons are transferred, one at a time, from the aminoquinol TTQ to amicyanin. The first reoxidation step is catalytically gated by deprotonation of the aminoquinol and is activated by specific monovalent cations.[14,15] This step occurs in the crystal but is too fast to be resolved by using single crystal microspectrophotometry.[16]

The identification of the natural acceptor of electrons from amicyanin is still a matter of debate. During methylotrophic growth, three soluble *c*-type cytochromes are present in the *P. denitrificans* periplasm: the constitutive cytochrome *c*-550 and the two inducible cytochromes *c*-551i and *c*-553i.[17] Gene knockout experiments showed that the lack of cytochrome *c*-551i only affected the growth rate on methanol but not on methylamine, thus identifying this cytochrome as the physiological electron acceptor of methanol dehydrogenase.[18,19] However, cytochrome *c*-551i has also been shown to be the most efficient acceptor of electrons from amicyanin in vitro.[17] Cytochrome *c*-551i has been proposed to interact with the MADH−amicyanin binary complex,[11,20] and the occurrence of the ternary complex in solution was inferred from kinetic and thermodynamic studies.[21,22] In the crystalline ternary complex,[6,23] the mode of association of MADH and amicyanin was the same as in the binary complex. Possible paths for electron transfer through the protein matrix were proposed.[22,24]

Single crystal polarized absorption microspectrophotometric measurements have shown that the ternary complex is competent in transferring methylamine-derived electrons to the cytochrome heme.[25] However, the rate of electron transfer from the copper of amicyanin to the heme was found to be more than 4 orders of magnitude lower in the crystal than in solution,[16] a value close to that expected on the basis of the distance between the two centers observed in the crystal.[26,27] Furthermore, the absence of the amicyanin copper in crystals of the ternary complex, obtained with apoamicyanin, did not prevent slow reduction of the cytochrome, permitting direct electron transfer from the aminoquinol TTQ.[25] These findings raised the question whether the crystal structure is a suitable model to describe the intermolecular electron transfer among these proteins.

Two-dimensional NMR techniques offer the opportunity to investigate protein−protein interactions in solution rather than in the fixed lattice network. In a previous paper,[28] we reported an NMR study and determined the crystal structure of the binary complex between MADH and amicyanin from *P. versutus*. Phylogenetically, the Gram-negative bacteria *P. versutus* and *P. denitrificans* are closely related and share the common feature of methylotrophic growth, although some differences have been reported in the protein composition of the two redox chains.[29,30] We have been unable to grow crystals of a ternary complex when using MADH and amicyanin from *P. versutus* and cytochrome *c*-551i from *P. denitrificans*.

In the present study, we report the perturbation of amicyanin chemical shifts by the electron donor, MADH, and by the electron acceptor, cytochrome *c*-551i, all from *P. denitrificans*. Furthermore, we describe the effect of the presence of the enzyme on the interaction between the cupredoxin and the cytochrome. Perdeuteration and ^{15}N-labeling of amicyanin allowed us to identify the interface of amicyanin involved in the association with MADH in solution and to compare it to that observed in the crystal. The interaction of both free and MADH-bound amicyanin with cytochrome *c*-551i was similarly investigated. The results obtained by NMR spectroscopy experiments were complemented with oxygraphic activity assays to study the rates of the reaction between free and MADH-bound amicyanin with cytochrome *c*-551i. The results of both approaches were found to be consistent and provide evidence that free amicyanin and not the binary complex interacts with cytochrome *c*-551i.

Materials and Methods

Native Protein Expression and Purification. *P. denitrificans* (Pd1222) was incubated at 30 °C in a medium composed of methylamine hydrochloride (4.8 g/L), K_2HPO_4 (6 g/L), KH_2PO_4 (4 g/L), NH_4Cl (4 g/L), $MgSO_4 \cdot 7H_2O$ (0.25 g/L), citric acid (0.42 g/L), yeast extract (0.1 g/L), $NaHCO_3$ (1 g/L), and trace elements (0.5 mL/L). The trace elements solution was prepared according

(11) Gray, K. A.; Davidson, V. L.; Knaff, D. B. *J. Biol. Chem.* **1988**, *263*, 13987–90.
(12) Davidson, V. L.; Graichen, M. E.; Jones, L. H. *Biochim. Biophys. Acta* **1993**, *1144*, 39–45.
(13) McIntire, W. S.; Wemmer, D. E.; Chistoserdov, A.; Lidstrom, M. E. *Science* **1991**, *252*, 817–24.
(14) Bishop, G. R.; Davidson, V. L. *Biochemistry* **1995**, *34*, 12082–6.
(15) Bishop, G. R.; Davidson, V. L. *Biochemistry* **1997**, *36*, 13586–92.
(16) Ferrari, D.; Merli, A.; Peracchi, A.; Di Valentin, M.; Carbonera, D.; Rossi, G. L. *Biochim. Biophys. Acta* **2003**, *1647*, 337–42.
(17) Husain, M.; Davidson, V. L. *J. Biol. Chem.* **1986**, *261*, 8577–80.
(18) Van Spanning, R. J.; Wansell, C. W.; Reijnders, W. N.; Harms, N.; Ras, J.; Oltmann, L. F.; Stouthamer, A. H. *J. Bacteriol.* **1991**, *173*, 6962–70.
(19) Long, A. R.; Anthony, C. *J. Gen. Microbiol.* **1991**, *137*, 415–425.
(20) Gray, K. A.; Knaff, D. B.; Husain, M.; Davidson, V. L. *FEBS Lett.* **1986**, *207*, 239–42.
(21) Davidson, V. L.; Jones, L. H. *J. Biol. Chem.* **1995**, *270*, 23941–3.
(22) Davidson, V. L.; Jones, L. H. *Biochemistry* **1996**, *35*, 8120–5.
(23) Chen, L.; Mathews, F. S.; Davidson, V. L.; Tegoni, M.; Rivetti, C.; Rossi, G. L. *Protein Sci.* **1993**, *2*, 147–54.
(24) Mathews, F. S.; Chen, Z.-W.; Durley, R. C. E.; Davidson, V. L.; Jones, L. H.; Graichen, M. E.; Hosler, J. H.; Merli, A.; Brodersen, D. E.; Rossi, G. L. Structural research on the methylamine dehydrogenase redox chain of Paracoccus denitrificans. In *Biological Electron-Transfer Chains: Genetics, Composition and Mode of Operation*; Canters, G. W., Vijgenboom, E., Eds.; Kluwer Academic Publishers: Dordrecht, Netherlands, 1998; pp 129−146.
(25) Merli, A.; Brodersen, D. E.; Morini, B.; Chen, Z.; Durley, R. C.; Mathews, F. S.; Davidson, V. L.; Rossi, G. L. *J. Biol. Chem.* **1996**, *271*, 9177–80.
(26) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265–322.
(27) Page, C. C.; Moser, C. C.; Chen, X.; Dutton, P. L. *Nature* **1999**, *402*, 47–52.
(28) Cavalieri, C.; Biermann, N.; Vlasie, M. D.; Einsle, O.; Merli, A.; Ferrari, D.; Rossi, G. L.; Ubbink, M. *Biochemistry* **2008**, *47*, 6560–6570.
(29) van Wielink, J. E.; Frank Jzn, J.; Duine, J. A. Electron transport from methylamine to oxygen in the Gram-negative bacterium *Thiobacillus versutus*. In *PPQ and Quinoproteins*; Jongejan, J. A., Duine, J. A., Eds.; Kluwer Academic Publishers: Dordrecht, Netherlands, 1989; pp 269−278.
(30) Ubbink, M.; Hunt, N. I.; Hill, H. A.; Canters, G. W. *Eur. J. Biochem.* **1994**, *222*, 561–71.

to ref 31. The cells were grown in a 30 L fermenter, keeping the pH at 6.6 and the oxygen concentration above 50% saturation. The culture was harvested at midlog phase ($OD_{600} = 1.6$) by centrifugation at 6000 rpm for 25 min at 4 °C. The cells were resuspended in 200 mL of 10 mM potassium phosphate (pH 7.5) containing 50 mg of PMSF and 100 mg of lysozyme and DNase, yielding a total volume of 300 mL, and lysed using a French pressure cell. Cell debris was removed by centrifugation at 6000 rpm for 20 min, and supernatant was further centrifuged at 40 000 rpm for 2 h. The crude extract was diluted to 1 L with 10 mM potassium phosphate (pH 7.5) and applied to a DEAE column, equilibrated with the same buffer. After being washed with six column volumes of buffer, proteins were eluted with a 0−500 mM NaCl linear gradient; amicyanin eluted in the first half of the run, and the cytochromes and MADH eluted in the second half. The cytochromes and MADH pools were desalted, applied to a Q column equilibrated with 10 mM potassium phosphate (pH 7.5), and eluted with a 0−500 mM NaCl linear gradient. The eluted proteins and the amicyanin pool from the DEAE column were further purified over a Superdex 75 size exclusion chromatography column, using the same buffer. The yields for a 30 L culture were MADH, 0.61 μmol; amicyanin, 0.73 μmol; cytochrome *c*-550, 0.23 μmol; cytochrome *c*-551i, 0.39 μmol. The protein purity, checked by SDS-PAGE and isoelectric focusing, was close to 100% for amicyanin and cytochrome *c*-550, and >95% for MADH and cytochrome *c*-551i. MADH concentrations in this article refer to the tetramer. These values are doubled in the kinetic analysis because MADH acts as a functional dimer.

Dodecyl maltoside-solubilized four-subunit oxidase was obtained essentially as previously detailed.[32]

Production of Isotope-Enriched Amicyanin. A pET28a plasmid, containing the amicyanin gene, was transformed into *Escherichia coli* BL21. Amicyanin was obtained by incubating the cells at 30 °C on M9 minimal medium[33] with $^{15}NH_4Cl$ (1 g/L) as the sole nitrogen source, CD_3COONa (5 g/L, for deuterated samples) or glucose (2 g/L, ^{12}C or uniformly labeled ^{13}C) as the sole carbon source, and D_2O (>99.8%) substituting H_2O in the case deuteration was required. Kanamycin was added to a concentration of 50 mg/L. Gene expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the OD_{600} was 0.7, and incubation was allowed to continue for 24 h at 22 °C. Amicyanin was purified as described previously.[28] The purity of the protein was determined by taking the ratio A_{280}/A_{596} (3.0 for the pure protein), and the yield was about 15 and 30 mg/L of culture for deuterated and protonated samples, respectively, estimated with $\varepsilon_{596} = 4610\ M^{-1}\ cm^{-1}$ for the oxidized protein. The molecular mass of recombinant unlabeled amicyanin (11 620.8) matched the theoretical mass (11 621.3); similarly, the expected molecular masses were observed for the labeled proteins. The estimated deuteration level of nonexchangeable protons was >99%.

Zinc Replacement. Copper was substituted with zinc according to the procedure described in ref 34. To remove unfolded protein, zinc amicyanin was applied to a HiTrap Q Sepharose Fast Flow (1 mL) column (GE Healthcare) equilibrated with 20 mM Hepes (pH 7.5). Elution was performed with a linear gradient (0−50%) of 1 M NaCl.

NMR Experiments. All NMR experiments were performed on a Bruker Avance DMX 600 MHz NMR spectrometer equipped with a TCI-Z-GRAD ATM cryoprobe. For the assignment of the backbone amide resonances of reduced Cu-amicyanin, HNCACB,[35] HNCO,[36] HN(CA)CO,[37] CC(CO)NH,[38] and [$^{15}N-^{1}H$] HSQC spectra were recorded at 300 K on a 2.0 mM $^{13}C-^{15}N$ labeled sample of amicyanin, in 10 mM potassium phosphate (pH 6.8), 5 mM sodium ascorbate, and 10% D_2O. Zn-substituted amicyanin was assigned on the basis of NOESY-[$^{15}N-^{1}H$] HSQC and TOCSY-[$^{15}N-^{1}H$] HSQC spectra on a 1.2 mM ^{15}N labeled sample in 20 mM potassium phosphate (pH 7.9) and 6% D_2O.

All chemical shift perturbation and titration experiments were performed in 20 mM Hepes (pH 7.6) and 6% D_2O and were carried out at 300 K. TROSY spectra[39] were acquired on a 100 μM $^{15}N-^{2}H-$Zn-amicyanin sample and on the same protein in the presence of 50 or 150 μM MADH. Small aliquots of cytochrome *c*-551i were added in four steps to the sample with the higher MADH concentration. For the cytochrome *c*-551i titration experiments with free amicyanin, a series of $^{15}N-^{1}H$ HSQC and 1D spectra of a 100 μM $^{15}N-$Zn-amicyanin sample were recorded. Cytochrome *c*-551i was reduced with sodium ascorbate.

Processing of NMR data was performed with Azara (http://www.ccpn.ac.uk/azara/). Backbone assignment of Cu(I)-amicyanin was performed using the semiautomated procedure in Ansig-for-Windows.[40] All nonproline residues were assigned except for Asn54 for which no signals were observed. The assignments have been deposited in the BMRB, entry 16741. Amides in Zn-substituted amicyanin were assigned by comparison with Cu(I)-amicyanin and confirmed by analysis of the NOESY-[$^{15}N-^{1}H$] HSQC and TOCSY-[$^{15}N-^{1}H$] HSQC spectra. In this case, four residues were not assigned: His53, Asn54, Phe57, and His95. The assignments have been deposited under entry 16740. TROSY spectra were compared using Ansig-for-Windows. Free amicyanin resonances in the titration and chemical shift perturbation experiments were assigned by comparison with the previously assigned spectra. All nonproline residues were assigned, except for His53, Asn54, Phe57, Met71, His95 in the TROSY experiments and the same residues as well as Asp1, Ala12, Ala66, Leu67 in the HSQC spectra. Signals of the MADH-bound form of amicyanin were assigned by comparison with the free protein. For those residues that could not be assigned, a minimum shift was determined, based on the closest unassigned peak.

Chemical shift changes ($\Delta\delta$) of both amide nitrogens and protons observed during the titration were plotted against the cytochrome *c*-551i/amicyanin molar ratio (R), and the curves were fitted to a one-site binding model with the equations:

$$\Delta\delta = \frac{1}{2}\Delta\delta^{max}\left(Q - \sqrt{Q^2 - 4R}\right)$$
$$Q = 1 + R + \frac{A_0R + C_0}{A_0C_0K_A} \qquad (1)$$

where A_0 is the initial concentration of amicyanin, C_0 is the stock concentration of the cytochrome, $\Delta\delta^{max}$ is the maximal chemical shift change (for 100% bound amicyanin), and K_A (M^{-1}) is the association constant.[41] A global nonlinear fit, performed in Origin 7.5 (OriginLab), produced a single K_A for each titration.

The weighted average chemical shift perturbation for each peak was given by:

(31) Ludwig, B. *Methods Enzymol.* **1986**, *126*, 153–9.
(32) Hendler, R. W.; Pardhasaradhi, K.; Reynafarje, B.; Ludwig, B. *Biophys. J.* **1991**, *60*, 415–23.
(33) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A laboratory manual*; Cold Spring Harbor Laboratory Press: Plainview, NY, 1989.
(34) Ubbink, M.; Lian, L. Y.; Modi, S.; Evans, P. A.; Bendall, D. S. *Eur. J. Biochem.* **1996**, *242*, 132–47.
(35) Wittekind, M.; Mueller, L. *J. Magn. Reson., Ser. B* **1993**, *101*, 201–205.
(36) Grzesiek, S.; Bax, A. *J. Magn. Reson.* **1992**, *96*, 432–440.
(37) Clubb, R. T.; Thanabal, V.; Wagner, G. *J. Magn. Reson.* **1992**, *97*, 213–217.
(38) Montelione, G. T.; Lyons, B. A.; Emerson, S. D.; Tashiro, M. *J. Am. Chem. Soc.* **1992**, *114*, 10974–10975.
(39) Pervushin, K.; Riek, R.; Wider, G.; Wuthrich, K. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 12366–71.
(40) Helgstrand, M.; Kraulis, P.; Allard, P.; Hard, T. *J. Biomol. NMR* **2000**, *18*, 329–36.
(41) Kannt, A.; Young, S.; Bendall, D. S. *Biochim. Biophys. Acta* **1996**, *1277*, 115–126.

$$\Delta\delta_{avg} = \sqrt{\frac{\Delta\delta H^2 + (\Delta\delta N/5)^2}{2}} \quad (2)$$

where $\Delta\delta$H and $\Delta\delta$N are the chemical shifts perturbations upon binding of the amide proton and nitrogen, respectively, extrapolated to 100% of the bound state.

Activity Assays. Enzyme activity assays were performed at 20 °C in a solution of 1 mM methylamine hydrochloride, 10 mM potassium phosphate (pH 7.5), 50 mM KCl, 1 mM EDTA, and 0.1% dodecyl maltoside with a Hansatech Instruments oxygraph equipped with a Clark electrode. The oxygraph was calibrated with sodium dithionite, and the reaction volume was 400 μL. The rate of oxygen consumption was determined by measuring the slope of concentration decrease. The theory of the kinetic analysis is described in the Supporting Information.

Results

Interaction between Amicyanin and MADH. To investigate the interaction in solution between *P. denitrificans* amicyanin and MADH, an NMR spectroscopy strategy was followed similar to that described for the *P. versutus* complex.[28] The amicyanin-encoding gene was overexpressed recombinantly in *E. coli*, and the protein was isotopically labeled. The NMR resonances of the backbone amides were assigned using standard three-dimensional heteronuclear experiments on a $^{13}C-^{15}N$-amicyanin sample. Then, MADH was titrated into a $^{15}N-^{2}H$-amicyanin sample, and the amide resonances were observed using TROSY experiments.[39] Zn^{II}-substituted amicyanin was used as a redox-inactive and nonparamagnetic mimic of the oxidized (Cu^{II}) protein, to avoid line broadening by electron transfer reactions or paramagnetic relaxation. Many signals were perturbed by MADH binding, and the exchange between bound and free Zn^{II}-amicyanin was in the slow regime for most resonances. Consequently, it was not possible to assign some of the resonances that were heavily perturbed in the bound state, and only a minimum chemical shift change could be determined. In Figure 1, the average amide perturbations ($\Delta\delta_{avg}$, eq 2) are plotted for the observed Zn^{II}-amicyanin residues and color-coded on the surface of the protein.[42] The perturbed residues are located in and around the hydrophobic patch of Zn^{II}-amicyanin, in proximity to the copper center, with the largest effects observed for methionines 28, 51, and 98, His 91, Cys 92, Thr 93, and Phe 97. The largest $\Delta\delta_{avg}$ values are observed for Zn^{II}-amicyanin residues that are also closest to MADH in the crystal structure of the binary[5] and ternary complexes,[6] as illustrated in Figure 1C. A comparison between Figure 1B and C indicates that the interaction between Zn^{II}-amicyanin and MADH in solution resembles that of amicyanin in the crystal. Also further away from the interface small chemical shift perturbations are observed. All these findings are similar to the results reported for the complex between Zn^{II}-amicyanin and MADH from *P. versutus*.[28]

Interaction between Amicyanin and Cytochrome *c*-551i. A ^{15}N-labeled sample of Zn^{II}-amicyanin was titrated with cytochrome *c*-551i, and complex formation was followed with NMR spectroscopy. HSQC spectra were acquired at each titration point. Many amide resonances of Zn^{II}-amicyanin exhibited small chemical shift perturbations that increased with the addition of more cytochrome. It is concluded that the two proteins form a complex and association and dissociation occur in the fast exchange regime on the NMR time scale. The perturbations were

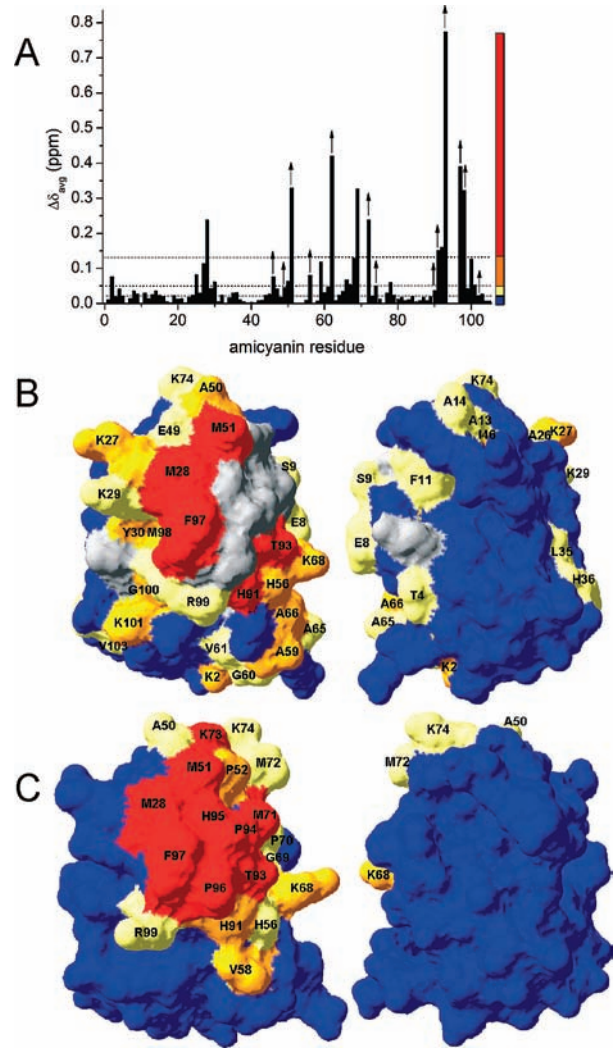


Figure 1. Interaction between Zn^{II}-amicyanin and MADH. (A) Histogram of the chemical shift perturbations of Zn^{II}-amicyanin amide groups upon complex formation with MADH. The arrows indicate the residues for which only a minimum shift could be determined (see text). (B) The amide chemical shift perturbations are color-coded on a surface representation of amicyanin (PDB entry 1AAN),[42] following the color bar shown in (A), with $\Delta\delta_{avg}$ (in ppm) < 0.025 in blue; 0.025 < $\Delta\delta_{avg}$ < 0.050 in yellow; 0.050< $\Delta\delta_{avg}$ < 0.130 in orange; and $\Delta\delta_{avg}$ > 0.130 in red; no data, in gray. (C) The interface observed in the crystal of the binary complex (PDB entry 1MDA, chain A),[5] with residues within 4, 5, and 6 Å of MADH in red, orange, and yellow, respectively. In (B) and (C), the right-hand view is rotated by 180° around the vertical axis as compared to the one on the left.

used to derive the dissociation constant, K_d^{AC}, which is 80 (±4) μM (Figure 2A). Figure 2B presents the binding map for cytochrome *c*-551i on the surface of amicyanin. A comparison with Figure 1B shows that Zn^{II}-amicyanin employs largely the same binding site for the cytochrome and MADH. The $\Delta\delta_{avg}$ values are smaller in the case of the interaction with cytochrome *c*-551i. These values are the perturbations at the 100% bound state, thus representing an intrinsic property of the complex. Small $\Delta\delta_{avg}$ values have been shown to be related to dynamics in the complex, with the initial, dynamic state of the protein complex, called the encounter complex,[43] representing an important fraction of the complex.[44−47] It is therefore probable that in the Zn^{II}-amicyanin−cytochrome *c*-551i complex the

(42) Durley, R.; Chen, L.; Lim, L. W.; Mathews, F. S.; Davidson, V. L. *Protein Sci.* **1993**, *2*, 739–52.

(43) Ubbink, M. *FEBS Lett.* **2009**, *583*, 1060–6.

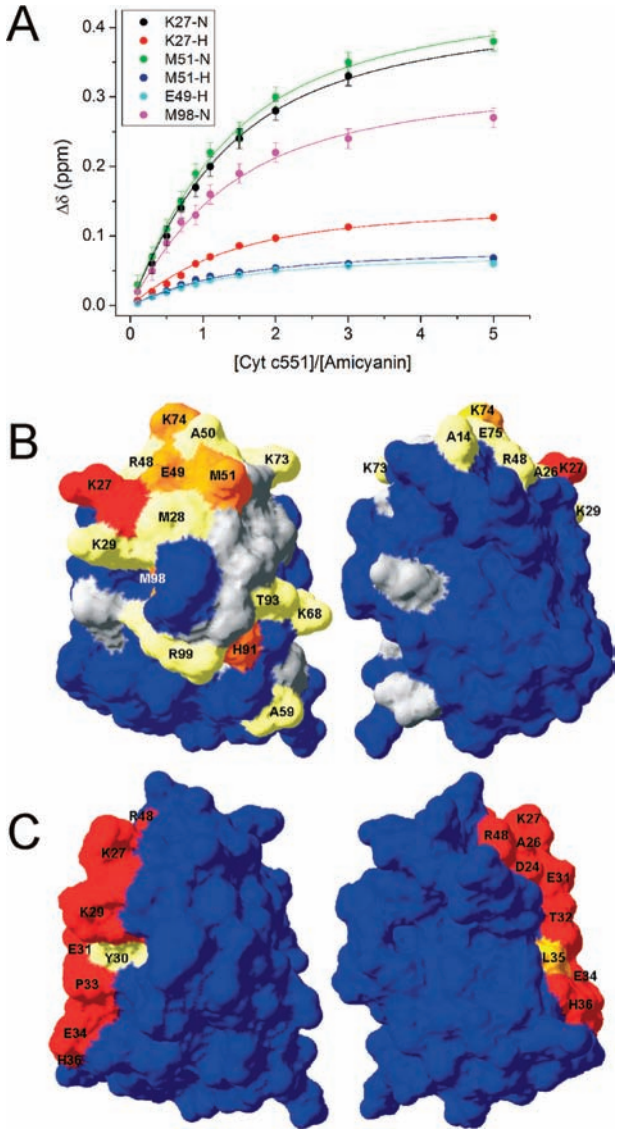


Figure 2. Interaction between Zn^{II}-amicyanin and cytochrome *c*-551i. (A) Chemical shift perturbations for ^{15}N and ^{1}H nuclei of several Zn^{II}-amicyanin residues are plotted against the ratio of cytochrome *c*-551i and Zn^{II}-amicyanin. The lines represent a global fit with a $K_d^{AC} = 80\ (\pm 4)\ \mu M$ to eq 1. (B) The perturbations are color-coded on the amicyanin surface (PDB entry 1AAN),[42] with $\Delta\delta_{avg}$ (in ppm) < 0.020 in blue; $0.020 < \Delta\delta_{avg} < 0.040$ in yellow; $0.040 < \Delta\delta_{avg} < 0.10$ in orange; and $\Delta\delta_{avg} > 0.10$ in red; no data, in gray. (C) The amicyanin interface with cytochrome *c*-551i in the crystal structure of the ternary complex (PDB entry 2MTA, chain C)[6] is shown, with residues within 4, 5, and 6 Å of cytochrome *c*-551i in red, orange, and yellow, respectively. In (B) and (C), the right-hand view is rotated by 180° around the vertical axis as compared to the one on the left.

interaction is less specific than in the Zn^{II}-amicyanin−MADH complex. The binding sites on amicyanin for cytochrome *c*-551i observed in solution and in the crystalline ternary complex[6] show little resemblance, as can be seen by comparing panels B and C of Figure 2. The interfaces overlap only at the edges, sharing residues Lys 27, Lys 29, and Arg 48.

(44) Worrall, J. A.; Liu, Y.; Crowley, P. B.; Nocek, J. M.; Hoffman, B. M.; Ubbink, M. *Biochemistry* **2002**, *41*, 11721–30.
(45) Worrall, J. A.; Reinle, W.; Bernhardt, R.; Ubbink, M. *Biochemistry* **2003**, *42*, 7068–76.
(46) Xu, X.; Reinle, W.; Hannemann, F.; Konarev, P. V.; Svergun, D. I.; Bernhardt, R.; Ubbink, M. *J. Am. Chem. Soc.* **2008**, *130*, 6395–403.
(47) Bashir, Q.; Volkov, A. N.; Ullmann, G. M.; Ubbink, M. *J. Am. Chem. Soc.* **2010**, *132*, 241–7.

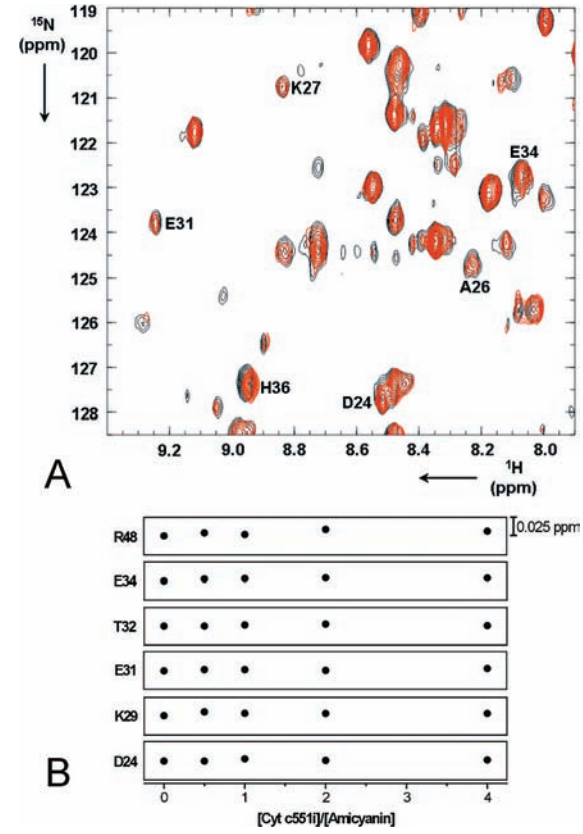
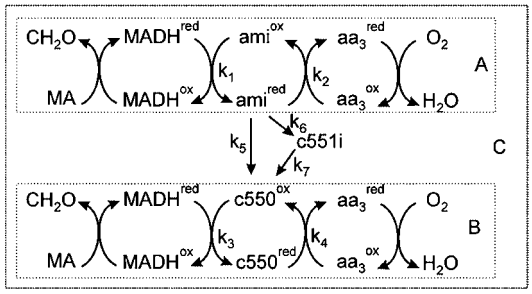


Figure 3. Interaction between the Zn^{II}-amicyanin−MADH binary complex and cytochrome *c*-551i. (A) Overlay of part of the TROSY spectra of the binary complex before (black) and after addition of 4 mol equiv of cytochrome *c*-551i (red), showing several resonances of residues in the interface between amicyanin and cytochrome *c*-551i in the crystal structure of the ternary complex (labeled). (B) The $\Delta\delta_{avg}$ is plotted for several of these residues in a titration of the binary complex with cytochrome *c*-551i. The bar represents 0.025 ppm, the significance limit used for the interaction between Zn^{II}-amicyanin and MADH in Figure 1.

Interaction between the Amicyanin−MADH Binary Complex and Cytochrome *c*-551i. To address the question whether the ternary complex is detectable in solution, cytochrome *c*-551i was titrated into a solution of the binary complex of $^{15}N-^{2}H$ labeled Zn^{II}-amicyanin (100 μM) and MADH (150 μM). The MADH tetramer has two binding sites for amicyanin, and, at this ratio, 99% or more of the Zn^{II}-amicyanin is bound. The highest cytochrome:Zn^{II}-amicyanin ratio was 4:1. TROSY spectra were acquired at each point in the titration to detect chemical shift perturbations of Zn^{II}-amicyanin amide resonances. No significant effects were observed for any signal. Several resonances in the region that represents the interface between amicyanin and cytochrome *c*-551i in the crystal structure of the ternary complex are shown in Figure 3. Even at the highest ratio, no perturbations are observed, except for a very small change for His 36, which can be attributed to a small pH effect, because this residue is known to be very sensitive to pH. These results strongly suggest that cytochrome *c*-551i does not associate to MADH-bound Zn^{II}-amicyanin in an appreciable amount in solution. The final concentrations of Zn^{II}-amicyanin and cytochrome in this experiment were 78 and 312 μM, respectively. The absence of significant chemical shift perturbations (>0.02 ppm) indicates, as a conservative estimate, that less than 10% of amicyanin is bound to cytochrome *c*-551i, that is, that $K_d \geq 2$ mM.

Scheme 1. Pathways of Electron Transport from Methylamine (MA) to Oxygen



Steady-State Kinetics of the Redox Chain. To establish whether cytochrome *c*-551i reacts with the binary complex or free amicyanin, a steady-state kinetics assay was used. The entire redox chain, including methylamine, MADH, amicyanin, the solubilized terminal oxidase cytochrome aa_3, and the relevant *c*-type cytochromes, was reconstituted in vitro in an oxygraph, and the oxygen consumption was used as a measure of activity.

To air-saturated buffer, methylamine, cytochrome aa_3 and MADH were added. No oxygen was consumed, indicating that reduced MADH cannot react with cytochrome aa_3. Upon addition of amicyanin, the oxygen concentration decreased, and the rate of oxygen consumption could be determined as a function of the amicyanin concentration (Scheme 1A and Figure 4A). The rate constant (k_2) is reported in nM of electrons per second, because the reduction of a single dioxygen molecule requires four successive electron donations by amicyanin. It was a surprising finding that amicyanin can react directly with cytochrome aa_3. This activity has not been reported before. Note that the reduction of amicyanin (k_1) was not the rate-limiting step, because the MADH concentration was sufficiently high (100 nM) to keep amicyanin reduced (see below).

Addition of cytochrome *c*-551i did not alter the rate of oxygen consumption (not shown), in agreement with the previous finding that electron transfer from cytochrome *c*-551i to membrane-bound oxidases is not appreciable.[48] If electrons are transferred to the cytochrome, under the conditions of this experiment, the reduced cytochrome would be a dead-end product.

On the other hand, addition of cytochrome *c*-550, constitutively present in the periplasm and known to be rapidly oxidized by cytochrome aa_3,[49] to a solution containing MADH, amicyanin, and cytochrome aa_3 enhances the rate of oxygen consumption. However, the effect is small, and a high concentration of cytochrome *c*-550 is required (Figure 4B, ■). The rate-limiting step in the electron transfer via cytochrome *c*-550 to oxygen is the reaction between reduced amicyanin and oxidized cytochrome *c*-550 (k_5 in Scheme 1C).

Interestingly, upon further addition of small amounts of cytochrome *c*-551i, oxygen consumption is drastically enhanced (Figure 4B, ▲), proving that cytochrome *c*-551i accelerates electron transfer from amicyanin to cytochrome *c*-550 (Scheme 1C, k_6 and k_7). The data show that the reactions are fast, despite the unfavorable difference in midpoint potentials.[20] All rate constants for the above-mentioned reactions are reported in Table 1.

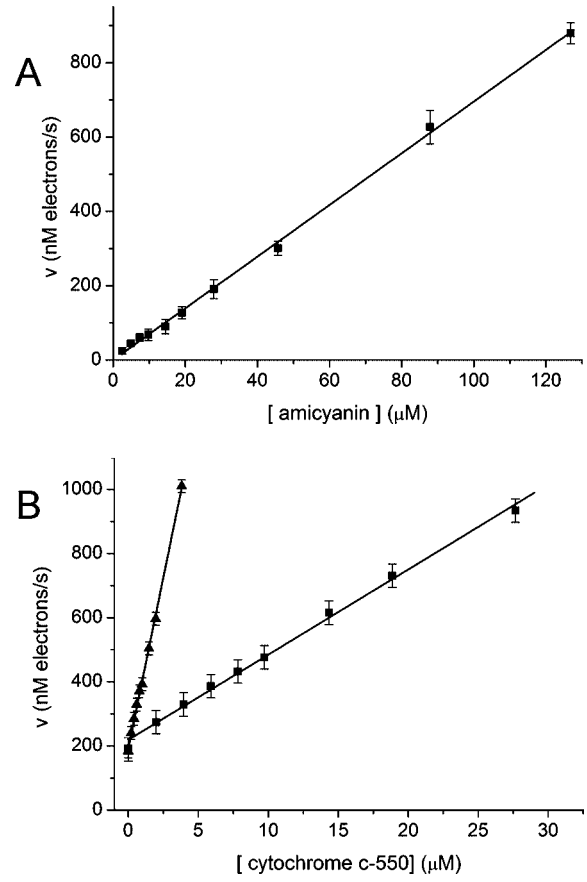


Figure 4. Steady-state kinetics. (A) The rate (v) of oxygen consumption (in nM electrons/s) is plotted against the concentration of amicyanin. MADH, 100 nM; methylamine, 1 mM; cytochrome aa_3, 40 nM. (B) The rate of oxygen consumption is plotted against the concentration of cytochrome *c*-550 in the presence of 10 μM amicyanin (■) or 10 μM amicyanin and 100 nM cytochrome *c*-551i (▲). Other components as in (A). The lines represent linear fits of the data, yielding k_2 (A) and k_2, k_5, k_7 (B) (see Supporting Information, eqs S-1, S-2, and S-3). The error bars represent the SD from two or three experiments.

Table 1. Reaction Parameters for the Methylamine Redox Chain[a]

parameter	
k_1	26 (±2) s^{-1}
k_{-1}	9 (±2)[b]
k_2	0.26[c]
k_3	0.0039 (±0.0003)
k_4	13 (±0.7)[d]
k_5	0.0028 (±0.0002)
k_6	~4
k_7	3.4 (±0.3)
K_d^{MA} (MADH-ami)	2.6 (±0.3)
K_d^{MC} (MADH-c550)	40 (±6)
K_d^{AC} (ami-c551i)	80 (±4)

[a] The rate constants (k) are defined in Scheme 1 and given in 10^6 M^{-1} s^{-1} (except k_1), and the dissociation constants (K_d) are given in μM. [b] k_{-1} represents the rate constant for association of amicyanin and MADH. [c] Some variation in k_2 was observed due to activity differences of the cytochrome aa_3 in different experiments. [d] Determined in a separate essay; results to be published elsewhere.

Inhibition by the Binary Complex. The oxygraphic assay was performed with an increasing concentration of MADH. At very low concentration of the enzyme, the concentration of the binary complex between amicyanin and MADH is negligible as compared to that of free amicyanin, whereas at the highest MADH concentrations nearly all amicyanin is bound.

(48) Davidson, V. L.; Kumar, M. A. *FEBS Lett.* **1989**, *245*, 271–3.
(49) Smith, L.; Newton, N.; Scholes, P. B. Reaction of cytochrome c oxidase of beef heart and Micrococcus denitrificans with mammalian and bacterial cytochromes c. In *Heme and Hemoproteins*; Chance, B., Estabrook, R., Yonetani, T., Eds.; Academic Press, Inc.: New York, 1966; pp 395−403.

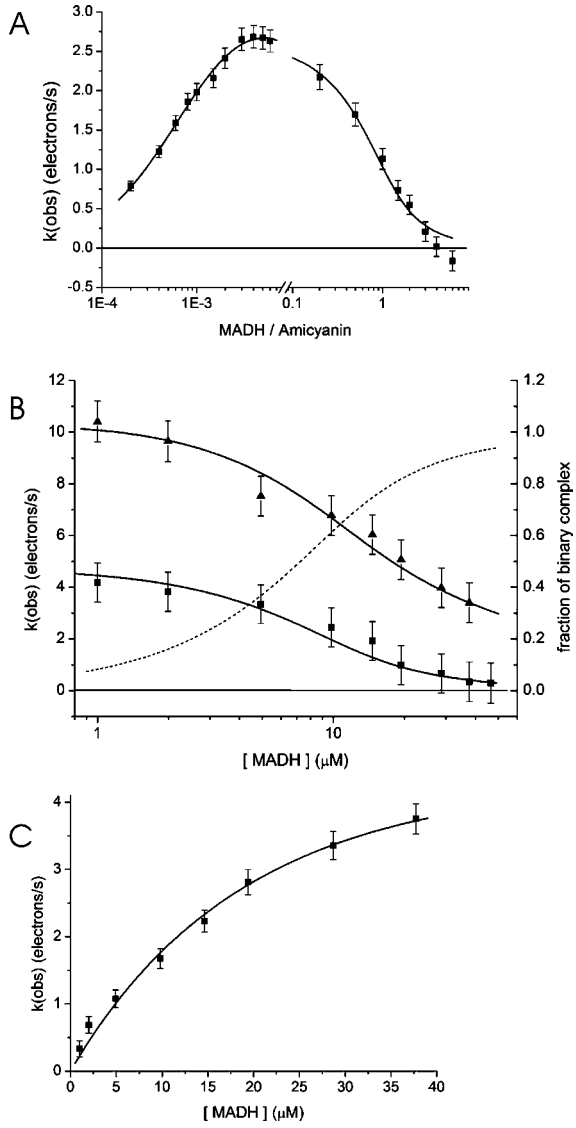


Figure 5. Rates of oxygen consumption at variable concentrations of MADH and constant concentrations of amicyanin (A and B, ■), amicyanin + cytochrome *c*-550 + cytochrome *c*-551i (B, ▲), and cytochrome *c*-550 (C). k_{obs} is the rate of oxygen consumption divided by the concentration of cytochrome aa_3. (A) k_{obs} is plotted against the ratio of MADH and amicyanin at low (left) and high values. Left: amicyanin, 20 μM; cytochrome aa_3, 100 nM; MADH from 2 to 60 nM. Right: amicyanin, 10 μM; cytochrome aa_3, 40 nM; MADH from 1 to 30 μM. (B) Amicyanin, 10 μM; cytochrome aa_3, 40 nM (both titrations) and cytochrome *c*-550, 1 μM and cytochrome *c*-551i, 100 nM (▲). (C) Cytochrome *c*-550, 2.5 μM; cytochrome aa_3, 40 nM. The solid lines represent fits to eqs S-4 (A and B, ■), S-6 (B, ▲), and S-5 (C). The dotted line in (B) indicates the fraction of amicyanin present in the binary complex (right axis). The parameters derived are k_1, k_{-1}, k_2, K_d^{MA} (A), k_6, k_7 (B), and k_3, K_d^{MC} (C). The error bars are based on the estimated experimental errors.

Figure 5A shows the rate of oxygen consumption as a function of the MADH: amicyanin ratio in the absence of *c*-type cytochromes. At very low ratios, the reduction of amicyanin by MADH is rate limiting. The steady-state reduction of amicyanin by MADH is independent of the amicyanin concentration, as was demonstrated in a spectrophotometric assay (Figure S1), suggesting that either the intracomplex reduction reaction or the dissociation of the MADH−amicyanin complex is the rate-limiting step, with a rate constant (k_1) of 20−30 s^{-1}. At a ratio of 0.003, equivalent to an MADH tetramer concentration of 30 nM, the electron transfer from amicyanin to cytochrome aa_3 already becomes the limiting step. At ratios higher than 0.1, the rate of oxygen reduction decreases with increasing MADH, indicating that free amicyanin but not the MADH−amicyanin complex can react with cytochrome aa_3. The curves have been fitted to the model in Scheme 1A, and the rate constants k_1, k_{-1}, and k_2 as well as the K_d^{MA} for the MADH−amicyanin complex are reported in Table 1. A detailed description of this and the following models and of the rate equations is provided in the Supporting Information. The fit yields a K_d^{MA} for the binary complex of 2.6 μM, consistent with the ratio of k_1 and k_{-1}, and in good agreement with the published value.[12]

To inquire whether cytochrome *c*-551i can react with the binary complex, the reaction was performed in the presence of fixed concentrations of amicyanin, cytochrome *c*-550, and cytochrome *c*-551i and increasing concentrations of MADH. As expected, a high rate of oxygen consumption was observed at low MADH concentrations, attributable to the catalytic effect of cytochrome *c*-551i on electron transfer between amicyanin and cytochrome *c*-550 (Figure 5B, ▲). At increasing MADH concentration, with progressive increase of the fraction of amicyanin in the binary complex (dotted line), oxygen consumption was significantly reduced. The rate does not go to zero, contrary to the results of the experiment with amicyanin alone (Figure 5B, ■), because of direct reduction of cytochrome *c*-550 by MADH. In fact, addition of cytochrome *c*-550 to the assay in the absence of amicyanin and cytochrome *c*-551i (Figure 5C) shows that this cytochrome can react slowly with MADH, in line with what has been reported for the *P. versutus* system.[30] A fit of the data according to Scheme 1B for the MADH−cytochrome *c*-550 binding yields a K_d^{MC} = 40 μM and $k_3 = 3.9 \times 10^3$ M^{-1} s^{-1} (Table 1).

It is evident that the activity decrease in the presence of both cytochromes anticorrelates with the fraction of amicyanin in the binary complex, strongly suggesting that cytochrome *c*-551i does not react with the binary complex. The results in Figure 5B were fitted to Scheme 1C, assuming that the MADH-bound amicyanin and the MADH-bound cytochrome *c*-550 fractions do not react (equations given in the Supporting Information). It should be noted that only two parameters, k_6 and k_7, were fitted to the data of Figure 5C, whereas all others were determined in the previously described experiments. Both constants are in the range of 10^6 M^{-1} s^{-1}. Scheme 1 contains the parameters fitted in the entire analysis (except k_{-1}, the association rate constant for amicyanin and MADH), and consistent values are obtained from the different data sets.

Discussion

Protein Interactions in Solution. The chemical shift perturbations observed for amide resonances upon complex formation between labeled Zn^{II}-amicyanin and MADH delineate the binding site. The hydrophobic patch and the surrounding positive charges show the largest effects, suggesting that these regions are part of the interface of the complex in solution, in good agreement with the site of interaction observed in the crystal structure of the binary complex[5] (Figure 1C). Additional small perturbations are seen for amides inside and on the surface of Zn^{II}-amicyanin, distant from the primary binding site. These results are very similar to those of the NMR study on the complex from *P. versutus*.[28] A careful comparison between the distant chemical shift changes and the crystal structures of free and bound Zn^{II}-amicyanin of that complex showed a correlation between tiny differences in the structures and the shift perturba-

tions, emphasizing the exquisite sensitivity of amide chemical shifts for structural changes.

Interestingly, Zn^{II}-amicyanin also interacts with cytochrome *c*-551i, albeit with much less affinity than for MADH. It uses about the same interaction site as for binding MADH, confirming an early speculation.[21] Potentially, it allows for fast electron transfer between the proteins, because a short distance between the heme and the copper (in the native Cu-amicyanin) is possible in this orientation. It has been observed frequently that small electron transfer proteins interact via a single site with multiple partners. For example, yeast cytochrome *c* uses the heme edge region and the surrounding positive side chains.[1,7,50−52] Similar observations have been made for other electron transfer reactions in *P. denitrificans*, for example, for the interactions of cytochrome *c*-550 and *c*-552 with the membrane-bound complexes cytochrome bc_1 and cytochrome aa_3.[53,54]

Such proteins often exhibit a dipolar charge distribution, which assists in successful docking by electrostatic preorientation.[43,55] Given the fact that both MADH and cytochrome *c*-551i are highly negative proteins, it is not surprising that amicyanin interacts via the same, positive site on its surface. The binding site shows only a small overlap at the edges with the binding site observed in the crystal structure of the ternary complex[6] (Figure 2C).

Amicyanin appears to lose its affinity for cytochrome *c*-551i when bound to MADH. No significant chemical shift perturbations for Zn^{II}-amicyanin residues are observed when the cytochrome is added to the binary complex. MADH occupies the primary binding site for the cytochrome on Zn^{II}-amicyanin, so it is not unexpected that no perturbations are observed there. In the crystal structure of the ternary complex, amicyanin binds cytochrome *c*-551i on a site more distant from copper. However, in solution no binding is observed, indicating that the affinity must be very weak, thus questioning whether such interaction would be of physiological significance. The ternary complex is formed reproducibly in the crystalline state, but the interface between amicyanin and the cytochrome is small (about 430 $Å^2$) and rather polar.[6]

Steady-State Kinetics. The measurement of oxygen consumption by cytochrome aa_3 makes it possible to reconstitute the methylamine redox chain in vitro, allowing for detailed manipulation of its composition. It was discovered that amicyanin can donate electrons directly to the terminal oxidase with a reasonable efficiency. This direct transfer may well be of relevance in vivo, because it has been demonstrated that *P. denitrificans* can still grow on methylamine after knockout of the genes for the cytochromes *c*-550, *c*-551i, and *c*-553i.[18] However, the addition of both cytochromes *c*-550 and *c*-551i enhances the oxygen consumption, indicating that also these proteins can have a role in the electron transfer process. Cytochrome *c*-550 reacts slowly with MADH and amicyanin, but fast with the oxidase. Cytochrome *c*-551i does not react with either MADH or cytochrome aa_3, but fast with both amicyanin and cytochrome *c*-550. Addition of both cytochromes thus enables rapid electron transfer from amicyanin, via cytochrome *c*-551i and cytochrome *c*-550, to the oxidase (Scheme 1).

By varying the concentration of MADH in solution, it is possible to manipulate the fractions of free and bound amicyanin. In this way, it was demonstrated that the MADH-bound amicyanin interacts neither with cytochrome aa_3 nor with cytochrome *c*-551i. Instead, dissociation of amicyanin from MADH is required for subsequent electron transfer reactions. Interestingly, the midpoint potential of cytochrome *c*-551i is lower than that of amicyanin, making the reduction of the former thermodynamically unfavorable. However, it is important to realize that the driving force that needs to be considered here is the one for the oxidation of methylamine to formaldehyde, combined with the reduction of dioxygen to water, which is very favorable. The proteins can all be considered as catalysts for this reaction. The small uphill step for electron transfer between amicyanin and cytochrome *c*-551i is irrelevant for the entire reaction, provided it does not limit the reaction rate. This rate is, in fact, quite fast, in agreement with the Marcus theory,[26] if it is assumed that the slightly unfavorable nuclear factor is combined with very efficient electronic coupling, due to a short copper-to-heme distance in the complex of cytochrome *c*-551i and amicyanin.

Davidson and Jones reported stopped-flow kinetic results[21] on the interaction of free and MADH-bound amicyanin with cytochrome *c*-551i. It was reported that free amicyanin reacts with the cytochrome within the dead time of the experiment, indicating very rapid electron transfer from amicyanin to cytochrome *c*-551i, despite the unfavorable midpoint potential difference. Given the short distance between the copper and heme cofactors that can be achieved when these proteins associate, the high rate would be in the correct range for electron transfer, assuming reasonable values for the coupling parameters, with $\beta = 1-1.2$ $Å^{-1}$ [56] or a packing density of the protein atoms $\rho = 0.85-0.90$.[27] More complete reduction, at a much lower rate, was observed when cytochrome *c*-551i was added to a mixture of amicyanin and MADH, such that most amicyanin is in the binary complex. It was concluded that in this experiment the cytochrome must react with the binary complex. The lower midpoint potential of amicyanin in the complex would allow for reduction of the cytochrome to a larger extent. The low reduction rate was explained by the long distance between the copper and the heme in the ternary complex. However, to account for the observed rate of electron transfer over the distance of 24.7 Å observed between the copper and heme in the crystal structure of the ternary complex, a very unusual coupling parameter of $\beta = 0.7$ $Å^{-1}$, corresponding to $\rho = 1.35$, would be required.[22] This decay factor β is lower than those observed for donor−acceptor complexes covalently coupled through a xylyl bridge,[56] and the packing density for a fully packed medium in a protein is $\rho = 1$.[27]

An alternative explanation of these results is possible. The more extensive reduction of the cytochrome is caused by the presence of reduced MADH, which pushes the equilibrium between oxidized and reduced cytochrome *c*-551i toward the latter. The lower reduction rate also does not necessarily imply a reaction with the binary complex. A different explanation, in

(50) Worrall, J. A.; Kolczak, U.; Canters, G. W.; Ubbink, M. *Biochemistry* **2001**, *40*, 7069–76.
(51) Volkov, A. N.; Worrall, J. A.; Holtzmann, E.; Ubbink, M. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 18945–50.
(52) Volkov, A. N.; Ferrari, D.; Worrall, J. A.; Bonvin, A. M.; Ubbink, M. *Protein Sci.* **2005**, *14*, 799–811.
(53) Maneg, O.; Malatesta, F.; Ludwig, B.; Drosou, V. *Biochim. Biophys. Acta* **2004**, *1655*, 274–81.
(54) Janzon, J.; Eichhorn, A. C.; Ludwig, B.; Malatesta, F. *Biochim. Biophys. Acta* **2008**, *1777*, 250–9.
(55) Schreiber, G.; Haran, G.; Zhou, H. X. *Chem. Rev.* **2009**, *109*, 839–860.
(56) Gray, H. B.; Winkler, J. R. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 3534–9.

accord with our data, is that amicyanin dissociation from MADH is the rate-limiting step. The reported value of 40−100 s^{-1} would be in the correct range. Our kinetic data yield a value of 26 s^{-1} for the rate-limiting step of steady state amicyanin reduction (k_1 in Table 1, a similar value is obtained from a spectrophotometric assay, Figure S1). We propose that this step represents the dissociation of the binary complex. Consistent with this proposal is that our NMR experiments indicate a slow-exchange regime. Alternatively, the much lower concentration of free amicyanin due to formation of the binary complex could serve as an explanation for the low rate.

Concluding Remarks

The NMR experiments and the steady-state kinetic data in this work strongly support a ping-pong interaction mode, in which amicyanin binds first to MADH to be reduced and then dissociates from MADH to react with cytochrome *c*-551i or cytochrome aa_3 via the same binding site. Electrons can reach the terminal oxidase via different routes, and it cannot be concluded that one specific interaction is preferred. In fact, in the periplasm of methylamine-grown *P. denitrificans*, also cytochrome *c*-553i and the membrane-bound cytochrome *c*-552 are found. The complex network that may connect all these electron transfer proteins is a subject of further study.

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Supporting Information Available: Theory and equations used for the kinetic analysis, as well as a figure showing the steady-state reduction of amicyanin in a spectrophotometric assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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