# Communication

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# Enzymatic and Electron Transfer Activities in Crystalline Protein Complexes*

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**Enzymatic and electron transfer activities have been studied by polarized absorption spectroscopy in single crystals of both binary and ternary complexes of methylamine dehydrogenase (MADH) with its redox partners. Within the crystals, MADH oxidizes methylamine, and the electrons are passed from the reduced tryptophan tryptophylquinone (TTQ) cofactor to the copper of amicyanin and to the heme of cytochrome $c_{551i}$ via amicyanin. The equilibrium distribution of electrons among the cofactors, and the rate of heme reduction after reaction with substrate, are both dependent on pH. The presence of copper in the ternary complex is not absolutely required for electron transfer from TTQ to heme, but its presence greatly enhances the rate of electron flow to the heme.**

Specific protein recognition is of the utmost importance for all biological systems both for regulation and for transfer of information, metabolites, and other components of living systems. For electron transfer, such recognition is needed for proper alignment of donor and acceptor molecules to achieve efficiency and to prevent energy loss through chance encounter leading to misdirected electron flow or abortive complex formation (1, 2). Several models for the interaction of protein partners have been developed, largely based on complementarity of surface charge or surface topology (3–5). However, direct observation of electron transfer complexes in the crystalline state between weakly associating partners has been reported in only three instances. These are a complex between cytochrome *c* and cytochrome *c* peroxidase (6), a complex between methylamine dehydrogenase (MADH)[1] and amicyanin (7), and a ternary complex between the latter complex and cytochrome $c_{551i}$ (8). Although these complexes provide much detailed structural information about the interacting surfaces and arrangement of cofactors between and among the partners, and suggest potential pathways for electrons to flow during transfer, questions about their physiological relevance and catalytic competence do arise. To address these questions, we have undertaken a single crystal polarized absorption study of the reactivity of the MADH-amicyanin and MADH-amicyanin-cytochrome $c_{551i}$ complexes in their crystalline states.

Polarized absorption microspectrophotometry can be a useful tool to probe the redox properties of proteins in the solid state. For example, earlier microspectrophotometric measurements showed that cytochrome *c* can diffuse into crystals of yeast flavocytochrome $b_2$ (L-lactate:cytochrome-*c* oxidoreductase) to form a reversible and functionally competent complex (9). In the present study, spectra have been recorded of crystals of the MADH binary and ternary complexes prepared using either copper-containing amicyanin or copper-free apoamicyanin. The crystals of these holo- and apocomplexes are isomorphous (10, 11). This isomorphism is very advantageous for these microspectrophotometry studies since it provides an internal control, allowing studies of the reactions of MADH in these crystalline complexes with and without the possibility of electron transfer through the copper atom. Furthermore, the spectral components of the prosthetic groups in this system can be better resolved when compared in the presence and absence of copper. Thus, this method would be well suited to test whether the arrangements of electron transfer partners observed in the two types of crystal lattice are competent for electron transfer and do not represent merely favorable but accidental crystal contacts.

MADH catalyzes the oxidation of methylamine in the periplasm of many methylotrophic and autotrophic bacteria to form ammonia and formaldehyde concomitant with the two-electron reduction of its redox cofactor tryptophan tryptophylquinone (TTQ) (12). In the autotrophs, the electrons are subsequently passed to a type I copper protein, amicyanin, then to one or more *c*-type cytochromes and finally to a membrane-bound cytochrome oxidase. For *Paracoccus denitrificans*, cytochrome $c_{551i}$ has been shown *in vitro* to accept electrons from a complex between MADH and amicyanin (13). The existence of this complex has been further demonstrated by chemical cross-linking and steady state kinetic analysis (14, 15). Rates of the electron transfer reactions between redox centers in the binary and ternary complex in solution have been measured by stopped-flow spectroscopy. The rate for heme reduction by Cu(I) in the ternary complex is 50–100 $s^{-1}$ at 30 °C (16). The rate for the reduction of copper by MADH in the binary complex is highly dependent on temperature, reaction conditions, and whether the substrate-derived amino group remains bound to reduced TTQ. Measured rates vary from 5 to several hundred per s (17–19).

MADH is an $H_2L_2$ heterotetramer with subunit molecular masses of 47 kDa and 15 kDa. The TTQ, located in the small subunit, is derived from two tryptophan side chains which are cross-linked and further modified to contain an orthoquinone function through a post-translational modification (20, 21). The

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[1] The abbreviations used are: MADH, methylamine dehydrogenase; TTQ, tryptophan tryptophylquinone.



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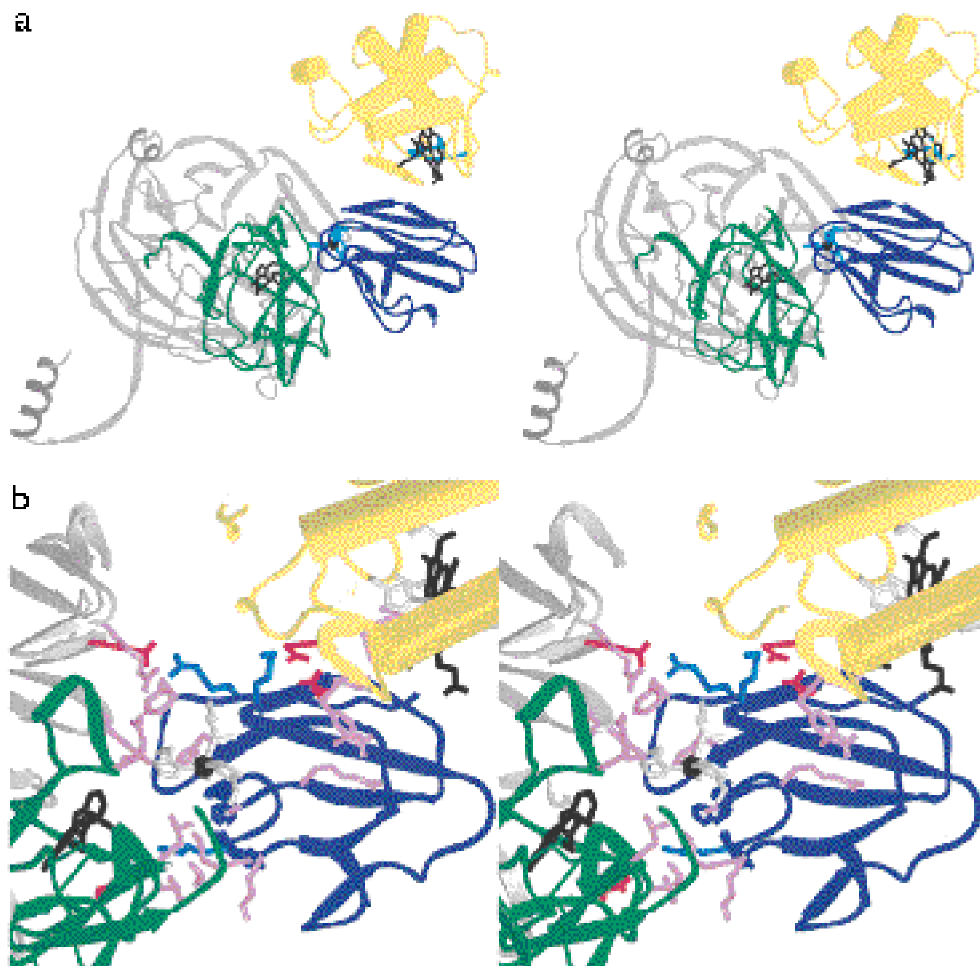


FIG. 1. *a*, stereo ribbon diagram of the ternary complex between MADH (half the heterotetramer), amicyanin, and cytochrome $c_{551i}$. The MADH H and L subunits are shown in *gray* and *green* and amicyanin and cytochrome are shown in *blue* and *yellow*, respectively. The three cofactors, TTQ, copper, and heme, are drawn in *black*, and the copper and heme ligands are highlighted in *light blue*. The quinone oxygens of TTQ lie close to the center of the β-disk of the H subunit whereas the second tryptophan ring is exposed to the MADH surface close to the copper site of amicyanin. The cytochrome approach is made to the twisted β-strand, which is shared between the two β-sheets, of amicyanin and does not involve MADH. This diagram was prepared using the molecular graphics program SETOR (32). *b*, this stereoview focuses on the two interfaces, one between amicyanin and MADH and the other between amicyanin and the cytochrome. The color scheme for the protein chains and cofactors is the same as in *a*. The copper and heme ligands are in *light gray*. Most of the interactions between MADH and amicyanin involve nonpolar groups, whereas the interactions between amicyanin and the cytochrome involve main chain hydrogen bonds and a greater number of polar side chains. Nonpolar groups or polar groups whose aliphatic portions are within an interface are shown in *pink*; polar residues which may be involved in salt bridges or hydrogen bonds are shown as *red* for acidic and *blue* for basic. This diagram was prepared using the molecular graphics program SETOR (32).

amicyanin has a molecular mass of 12.5 kDa and the cytochrome a molecular mass of 17.5 kDa. In the crystalline binary complex, one molecule of amicyanin is bound to each half of the MADH heterotetramer in an identical manner. In the crystalline ternary complex (Fig. 1*a*), the MADH and amicyanin are related in the same way as in the binary complex. The cytochrome binds to amicyanin at the hinge of the β-clamshell on the other side from the MADH binding site. The MADH-amicyanin interface is formed by a concave surface on the MADH molecule and a convex surface on the amicyanin. The latter is made up of one edge of the histidine ligand of the copper atom surrounded by a patch of 7 hydrophobic surface residues (Fig. 1*b*). The MADH interface contains the nonquinolated tryptophan of TTQ surrounded by approximately 8 hydrophobic residues from the small subunit and 4 from the large subunit. The quinolated tryptophan of TTQ is located in the interior of the light subunit at the enzyme active site. The distance between the O-6 quinone of TTQ and the copper is approximately 16 Å while the closest point on TTQ, Cε2, of the second tryptophan is about 9 Å from the copper. The amicyanin-cytochrome $c_{551i}$ interface is largely hydrophilic in nature, containing several ionic interactions, and is smaller than that between MADH and amicyanin (Fig. 1*b*). The iron of the heme and the copper of amicyanin are separated by approximately 25 Å and the distance from the O-6 quinone of TTQ to the iron is approximately 40 Å.

## EXPERIMENTAL PROCEDURES

*Polarized Absorption Spectra*—The methodology of polarized absorption spectroscopy of single crystals is well established (22–24). The spectra in this study were recorded using a Zeiss MPM800 microspectrophotometer. The crystals were placed in a flow cell with quartz windows. Tetragonal crystals of the binary complexes (space group $P4_12_12$) (10) grew as rhomboidal sections of a bipyrimid with the *c* axis as the longer diagonal of the observed (110) face. Absorption of the incident plane-polarized light was recorded with the beam directed normal to this face and the electric vector oriented parallel or perpendicular to the *c* axis; the absorption obeyed the Beer-Lambert law. Orthorhombic crystals of the ternary complex (space group $C222_1$) (11) grew flattened on their (101) face with the *b* axis parallel to the long edge of the crystal. The spectral data were recorded with the electric vector parallel or perpendicular to the *b* axis, both being extinction directions. The isotropic spectrum of the tetragonal binary complex crystals was calculated as $A_{iso} = (A_\parallel + 2A_\perp)/3$ where $A_\parallel$ and $A_\perp$ are the absorbances when the electric vector is parallel and perpendicular, respectively, to *c*. To compute the isotropic spectrum of the ternary complex would require that polarized spectra be measured in three

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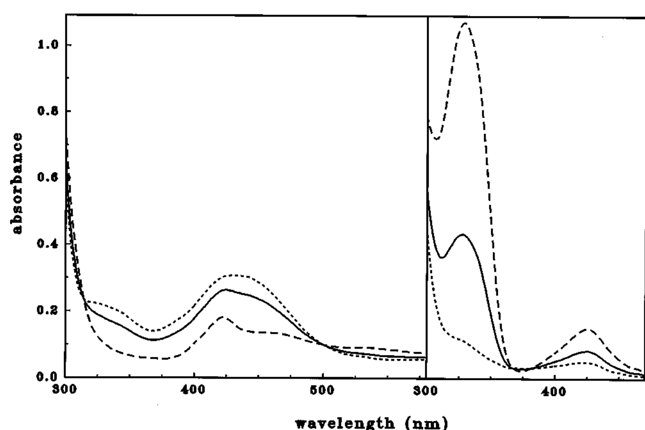


FIG. 2. **Polarized absorption spectra of a single crystal (0.1 × 0.05 × 0.01 mm) of the binary complex between MADH and apoamicyanin recorded at pH 7.5.** In the *left panel* are the spectra of the crystal oxidized by 3 mM potassium ferricyanide (since crystals in their native mother liquor were found to be partially reduced). In the *right panel* are spectra of the reduced crystal approximately 5 min after addition of 0.2 mM methylamine. The spectra shown are for the electric vector polarized parallel (*dashed lines*) and perpendicular (*dotted lines*) to the crystallographic *c* axis. The isotropic equivalent spectra are also shown (*solid lines*). The isosbestic points in the MADH spectrum upon reduction are within 2–3 nm of their values in solution under comparable conditions. After complete reduction of MADH, a residual peak at approximately 425 nm wavelength, similar to that of the semiquinone form of TTQ, remains. This peak cannot be eliminated by treatment with dithionite or other reducing agents and may represent a minor fraction of the enzyme which has been irreversibly modified.

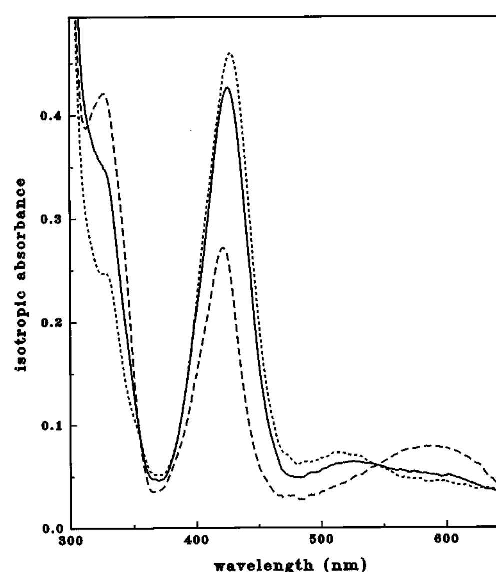


FIG. 3. **Isotropic absorption spectrum of a single crystal of the binary complex between MADH and amicyanin approximately 5 min after reduction by 0.2 mM methylamine, observed at pH 5.7 (*dashed line*), pH 7.5 (*solid line*), and pH 9.0 (*dotted line*).** The three absorption maxima present in the spectra occur at approximately 330 nm, 420 nm, and 590 nm and correspond to the reduced TTQ, TTQ semiquinone, and Cu(II), respectively. The relative absorbances near 420 nm are not quantitative since they may contain some contribution from a redox inactive species present in the sample (see legend to Fig. 2).

principal directions from at least two orientations; the plate-like crystals could be examined in only one orientation, precluding such a calculation. Appearance and disappearance of oxidized, semiquinone, and reduced signals from TTQ in the binary complex and of oxidized and reduced cytochrome in the ternary complex could be readily followed. The main absorption band for copper and some bands of the oxidized and semiquinone TTQ overlapped and could not be resolved easily.

*Protein Preparation*—Apoamicyanin was prepared by removal of the copper from purified amicyanin using sodium cyanide (25). MADH, amicyanin, and cytochrome $c_{551i}$ were prepared and purified as described previously (26–28). Crystals of the binary and ternary complexes with either holo- or apoamicyanin were prepared by vapor diffusion after mixing the protein components, in approximate molar ratios 1:3 and 1:3:3, respectively, with an equal volume of the reservoir solution. The equilibration reservoir contained 2.4 to 2.6 M monobasic sodium/dibasic potassium phosphate buffer in ratios 80:20 to 70:30 giving pH values of 5.5–5.7.

## RESULTS AND DISCUSSION

*Reduction of the Binary Complex*—The polarized absorption spectra show that crystals of the apobinary complex are reduced fully by methylamine and exhibit changes consistent with 2-electron reduction of TTQ as observed in solution (29) (Fig. 2). Minor variations can be attributed to differences in ionic strength, composition of the medium, and pH (30). Thus, the crystal lattice appears to have little effect on the electronic properties of TTQ in the enzyme, as would be expected. More importantly, the presence of apoamicyanin in complex with MADH also has only a minor effect on its spectrum. The polarization ratio ($A_{\parallel}/A_{\perp}$) for MADH changes dramatically upon reduction of the oxidized TTQ cofactor. In the oxidized crystal, $A_{\perp}$ is generally 2-fold larger than $A_{\parallel}$, while the reverse is true for the reduced crystal. This indicates that the direction of the transition dipole moment of TTQ changes when the enzyme is reduced as a result of changes in the electronic structure of TTQ and possibly of its orientation.

Addition of methylamine to crystals of the holobinary complex causes spectral changes indicating the formation of significant amounts of the semiquinone form of TTQ (Fig. 3). In these crystals, the semiquinone can only be formed by transfer of one electron from TTQ to the copper of amicyanin after the TTQ has first been reduced fully to the hydroquinone form by substrate. This demonstrates that MADH in the crystalline holobinary complex is competent both in catalysis and electron transfer. The amount of semiquinone formed is dependent upon pH. At pH 5.7, a large fraction of the TTQ remains reduced and a significant absorbance by Cu(II) can be observed, whereas at pH 9.0 the TTQ is mostly in the semiquinone form. Furthermore, after the reaction of the crystal with methylamine is complete, the ratio of semiquinone to reduced TTQ can be shifted reversibly by shifting the pH, suggesting that the difference between the redox potentials for the TTQ semiquinone/reduced couple and the $Cu^{2+}/Cu^{+}$ couple is pH-dependent.

The pH dependence of the electron distribution between TTQ and the copper in crystals of the binary complex may result from two factors. One is stabilization of the TTQ semiquinone at high pH. This has been demonstrated by solution studies involving titration of MADH with substoichiometric amounts of methylamine at low ionic strength; redistribution of electrons between reduced and oxidized MADH to form semiquinone was found to occur at high pH (31). This stabilization could arise, for example, by dissociation of a proton from reduced TTQ but not from the semiquinone form at high pH. The other factor could be a pH dependence of the redox potential of amicyanin when it is complexed with MADH. It is known that the amicyanin redox potential drops by 73 mV when in complex with MADH (13). Reduced amicyanin in the crystalline state has been found to undergo a conformational change at low pH (below about pH 6) resulting from protonation of the exposed histidine ligand to copper with rotation of 180° about the $C_{\beta}$-$C_{\alpha}$ bond and movement away from the copper into solution.[2] In the binary complex, such a histidine flip would move the imidazole

[2] L. M. Cunane, R. C. E. Durley, Z. Chen, W. Tarng, and F. S. Mathews, unpublished crystallographic results.

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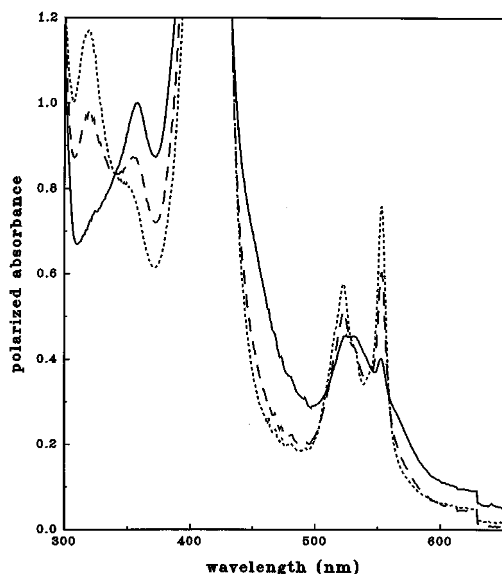


FIG. 4. **Polarized absorption spectrum of a single crystal (0.1 × 0.1 × 0.03 mm) of the ternary complex between MADH, amicyanin, and cytochrome $c_{551i}$ recorded at pH 7.5.** Spectra are recorded for the native complex (*solid line*) and at 13 min (*dashed line*) and 60 min (*dotted line*) after addition of 0.2 mM methylamine. For this spectrum, the electric vector of the polarized light is perpendicular to the crystallographic *b* axis. When the electric vector is aligned parallel to the *b* axis, the spectrum is weak and relatively featureless, consistent with the fact that the planes of the heme groups which contain the principal transition dipole moments are approximately perpendicular to the *b* axis (11). Since the native crystal was not pretreated with ferricyanide, it contains a small amount of reduced heme.

ring about 0.7 Å closer to MADH, according to a simple model building experiment, promoting disruption of the MADH-amicyanin interface. This would destabilize the reduced form of amicyanin in the complex and diminish its redox potential with respect to TTQ at lower pH.

*Reduction of the Ternary Complex*—Within the holoternary complex, heme reduction occurs when crystals are treated with methylamine at pH 7.5 (Fig. 4). The rate of heme reduction is dependent on pH. Even after several days, there had been little reduction of heme when substrate was added at pH 5.7. This is consistent with the observation that electron transfer from reduced TTQ to copper in the crystals of the binary complex is much less favorable at that pH. At pH 6.5, many hours are required to obtain even partial heme reduction while at pH 9.0 reduction is nearly complete within 35 min.

When methylamine is added to the apoternary complex crystal at pH 7.5, very little heme reduction occurs after 1 h, but is nearly complete after 4 days. Conversely, no reduction of the heme occurs at pH 5.7. Since the overall distance from TTQ to heme is the same in the holoternary and apoternary complexes and pathways for electron transfer from TTQ to heme are undoubtedly available within the apoternary complex, the fundamental question arises as to how the pH and the presence of copper in the ternary complex can enhance, by some orders of magnitude, the rate of the electron transfer reaction from TTQ to the heme in the crystalline state.

The reaction rates in these crystalline complexes may be limited by substrate diffusion or by one or more of the catalytic steps and may depend on the local environments of the protein molecules which differ considerably from those in solution. In the case of the holobinary complex, the rate does not appear to be limited by electron transfer from TTQ to copper since no accumulation of reduced TTQ is observed before semiquinone formation. In the case of the ternary complex, the copper to heme electron transfer rate may be rate-limiting. The challenge for the future is to compare rates in the crystal and in solution and to explain the means by which the observed structure might achieve such greater efficiency in solution.

These studies show that the holobinary and the holoternary complexes are competent both for substrate oxidation and for electron transfer. The results do not prove that the orientation of proteins in the crystallized complexes are exactly those which occur *in vivo* or that this is the only possible orientation for these proteins. The present studies do, however, clearly demonstrate that catalysis and long range electron transfer from TTQ to copper and from TTQ to heme via copper can and do occur in a predictable manner when the proteins are present in this orientation.

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