Complex Formation with Methylamine Dehydrogenase Affects the Pathway of Electron Transfer from Amicyanin to Cytochrome c-551i

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Methylamine dehydrogenase (MADH), amicyanin, and cytochrome c-551i are soluble redox proteins that form a complex in solution (Chen, L., Durley, R., Mathews, F. S., and Davidson, V. L. (1994) Science 264, 86-90), which is required for the physiologic electron transfer from the tryptophan tryptophylquinone cofactor of MADH to heme via the copper center of amicyanin. The reduction of cytochrome by amicyanin within the complex in solution has been demonstrated using rapid scanning stopped-flow spectroscopy. Electron transfer from free, uncomplexed, amicyanin to cytochrome c-551i occurs much more rapidly but only to a very small extent because the reaction is thermodynamically much less favorable when amicyanin is not associated with MADH (Gray, K. A., Davidson, V. L., and Knaff, D. B. (1988) J. Biol. Chem. 263, 13987-13990). These kinetic data suggest that amicyanin binding to cytochrome c-551i occurs at different sites when amicyanin is free and when it is in complex with MADH. A model for the interactions of these proteins is presented.

The electron transfer complex of methylamine dehydrogenase (MADH), amicyanin, and cytochrome c-551i from Paracoccus denitrificans is the only such physiologic complex of three weakly associating redox proteins for which a detailed crystal structure is available (Chen et al., 1994). Only two other two-protein electron transfer complexes have been structurally characterized, the binary complex of MADH and amicyanin (Chen et al., 1992) and the complex of cytochrome c peroxidase and cytochrome c (Pelletier and Kraut, 1992), MADH (Davidson, 1993) catalyzes the oxidative deamination of methylamine to formaldehyde plus ammonia and possesses the tryptophan tryptophylquinone (MIntire et al., 1991) prosthetic group, which participates in catalysis and electron transfer. The physiologic electron acceptor for most MADHs is amicyanin (Husain and Davidson, 1985), a type I copper protein. In P. denitrificans, amicyanin is absolutely required to mediate the transfer of electrons from MADH to the respiratory chain via c-type cytochromes. This has been demonstrated in vitro (Husain and Davidson, 1986), and it was also shown that inactivation of amicyanin in vivo by means of gene replacement resulted in complete loss of the ability to grow on methylamine (Van Spanning et al., 1990). In vivo studies have shown that of the c-type cytochromes isolated from P. denitrificans, the most efficient electron acceptor for the MADH-amicyanin complex is cytochrome c-551i (Husain and Davidson, 1986).

From the crystal structure of the protein complex it is possible to infer the sites of protein-protein interaction and putative electron transfer pathways, which connect the redox centers. One would like to correlate this structural information with kinetic data on the electron transfer reactions that occur within the protein complex. Ideally, these kinetic studies would be performed using the unmodified proteins. In the case of a complex containing three redox-active proteins the design of such experiments and the interpretation of data are complicated by the possibility that multiple alternative reactions may occur in solution. We have previously characterized the electron transfer reaction from the tryptophan tryptophylquinone of MADH to amicyanin by transient kinetic analysis (Brooks and Davidson, 1994). The steady-state kinetics of the methylamine-dependent reduction of cytochrome c-551i by MADH and amicyanin have also been characterized (Davidson and Jones, 1991). In this paper we describe transient kinetic studies of the electron transfer reaction from amicyanin to cytochrome c-551i in which rapid scanning stopped-flow spectroscopy was used to facilitate the interpretation of the spectral changes associated with this reaction.

EXPERIMENTAL PROCEDURES

Previously described procedures were used to purify MADH (Davidson, 1990), amicyanin (Husain and Davidson, 1985), and cytochrome c-551i (Husain and Davidson, 1986) from P. denitrificans (ATCC 13543). MADH was reduced by addition of methylamine. Amicyanin was oxidized by addition of potassium ferricyanide. All reagents were obtained from commercial sources.

An On-Line Instruments (OLIS, Bogart, GA) RSM1000 stopped-flow rapid scanning spectrophotometer was used for kinetic measurements. All experiments were performed in 0.01 M potassium phosphate, pH 7.5, at 30°C. Protein concentrations were determined from the previously reported extinction coefficients for each protein (Husain and Davidson, 1985, 1986; Husain et al., 1987). Non-linear curve fitting of data was performed using either OLIS software or Sigma Plot 5.0 (Jandel Scientific).

RESULTS AND DISCUSSION

To examine this reaction in a system that contains three weakly associating proteins, the first consideration in using a rapid mixing technology is to determine what combination of what redox forms of the proteins should be mixed to initiate the reaction. In our experimental design, one mixing syringe contained oxidized cytochrome c-551i, and the other contained reduced MADH plus reduced amicyanin. The reduced forms of MADH and amicyanin were quite stable against reoxidation by air and so anaerobic conditions were not necessary. Our aim was to mix fully reduced complex with oxidized cytochrome c-551i so that the initial event detectable after mixing would be the reduction of the cytochrome by amicyanin. Control stopped-flow experiments confirmed previous steady-state observations (Husain and Davidson, 1986) that no reaction occurred be-
between reduced MADH and oxidized cytochrome c-551i in the absence of amicyanin. Furthermore, as discussed later, no redox reaction between free amicyanin and cytochrome is detectable during the time course of these experiments. To ensure that essentially all of the reduced amicyanin was in complex with MADH, experiments were performed at concentrations of MADH and amicyanin, which were well above the K_d for the MADH-amicyanin complex. The K_d value for the MADH-amicyanin complex has been previously determined to be approximately 4 μM (Davidson et al., 1993; Brooks and Davidson, 1994) under these buffer conditions.

Mixing of oxidized cytochrome c-551i with the reduced MADH-amicyanin complex resulted in spectral changes indicative of reduction of the cytochrome (Fig. 1A). For subsequent kinetic analyses the observed rate constant (k_{obs}) was determined from the absorbance change with time at 552 nm (Fig. 1B). Under these experimental pseudo-first order conditions, all absorbance changes could be fit to a single exponential. Essentially identical results were obtained when the reaction was monitored at 419 nm. These experiments were repeated with different concentrations of proteins, and the maximum k_{obs} ranged from 50 to 100 s^{-1}, depending upon reaction conditions.

In contrast to the results discussed above, when reduced amicyanin was mixed with oxidized cytochrome c-551i in the absence of MADH at the same concentrations and under identical conditions, no reduction of cytochrome was observed during the time course of the experiment (Fig. 2). This confirms that the absorbance changes detected in Fig. 1 are due solely to the reaction of cytochrome c-551i with amicyanin in complex with MADH. This is also consistent with previous observations that amicyanin is a very poor reductant for cytochrome c-551i in the absence of MADH because the ΔE_m (oxidation-reduction midpoint potential) value for this reaction of −104 mV is thermodynamically very unfavorable (Gray et al., 1986). It is known that the redox potential of amicyanin decreases by 73 mV on complex formation with MADH (Gray et al., 1988), thereby facilitating the electron transfer to the cytochrome.

It is interesting to note that the initial spectrum recorded in Fig. 2A indicated that some partial reduction of the cytochrome had occurred during the dead time for mixing (approximately 2–3 ms). This is because when the ratio of reduced amicyanin to oxidized cytochrome is sufficiently large, some reduction of the latter will be observed despite the unfavorable potential. Using Equation 1,

$$\Delta E_m = (RT \ln F) \log K_{eq}$$

(Eq. 1)

and the known extinction coefficients for cytochrome c-551i, we have calculated that the extent of cytochrome reduction in the absence of MADH is exactly what would be predicted given the
The observation that the electron transfer reaction from amicyanin in complex to cytochrome c-551 is much slower than the rate of reaction from free amicyanin is unexpected. Based solely on thermodynamic considerations, it should be much faster because the $\Delta E_m$ for the reaction is 73 mV more favorable. These results indicate that the parameter $k_{ET}$ influences electron transfer rates, is different for the reactions of cytochrome c-551 with amicyanin when it is free in solution and when it is in complex with MAdH. Electron transfer theory (Marcus and Sutin, 1985) predicts that the rate of an electron transfer reaction will depend upon not only the driving force and temperature but also the atomic reorganization energy ($\lambda$) and electronic coupling ($H_{AB}$). The latter parameter reflects the distance and nature of the intervening media between redox centers. The former describes the energy barrier for electron transfer, independent of the driving force. If the redox centers and pathway for electron transfer do not change, then one would not expect $H_{AB}$ or $\lambda$ to change significantly.

The most reasonable explanation for these results is that electron transfer reactions from free and complexed amicyanin are occurring over different pathways in the two reactions. This would definitely result in different values of $H_{AB}$ for the two reactions and could also result in different $\lambda$ values. This would be true if free and complexed amicyanin interacted with cytochrome c-551 at different sites (Fig. 3). In the ternary protein complex (Chen et al., 1994), the hydrophobic patch that surrounds the His$^{59}$ ligand to copper is the site of interaction with MAdH, and the site of interaction with cytochrome c-551 is a more hydrophilic region well separated from the hydrophobic patch. Cytochrome c-551i, unlike mammalian cytochrome c and bacterial cytochromes c$_2$, does not exhibit a positively charged region surrounding the exposed heme edge. On cytochrome c-551i, the region immediately surrounding the exposed heme edge is relatively hydrophobic. Cytochrome c-551i interacts in the ternary complex with amicyanin at a more hydrophilic region near the heme. It is possible that when amicyanin is free from MAdH it is able to interact with cytochrome c-551i in an orientation such that the hydrophobic patch surrounding the copper ligand and the hydrophobic region surrounding the heme are in contact. This would place the two redox centers in much closer proximity to each other than when they are in the ternary complex. This alternative orientation cannot be achieved in the ternary complex because amicyanin is using its hydrophobic patch to interact with MAdH. The minimization of electron transfer distance for the reaction of free amicyanin with cytochrome would lead to a huge enhancement in the rate of electron transfer (Marcus and Sutin, 1985), which would more than compensate for the less favorable driving force and explain why the electron transfer reaction is slower in the complex.

This model brings into question the physiologic relevance of the ternary complex. However, it must be noted that while the reaction of free amicyanin with cytochrome c-551i is much faster it is very unfavorable. In the absence of complex formation net electron transfer in the physiologic direction will only occur if free amicyanin is in vast excess of cytochrome c-551i. There is no evidence that this is true. During our purification of amicyanin and cytochrome c-551i from methylamine-grown cells, the molar yields of these two proteins are very similar. Thus, the electron transfer from amicyanin to cytochrome c-551i within the ternary complex is very likely the physiologic reaction.

It has been well established that amicyanin is an obligatory intermediate in coupling the oxidation of methylamine to respiration in P. denitrificans (Van Spanning et al., 1990). This raises the question of why nature would design a sequential electron transfer process that requires a thermodynamically unfavorable intermediate step and complex formation between weakly associating soluble proteins. It may be that specificity of the protein interactions is of greater concern than the speed at which the electron is transferred. The most critical concern may be that the electrons derived from the oxidation of soluble substrates be delivered to the appropriate location in the membrane-bound respiratory chain with maximum accuracy. If electron transfer rates were maximized at the expense of specificity then energy would be wasted. This is a factor that must be acknowledged when evaluating biologic electron transfer processes, especially those involving soluble redox proteins.

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FIG. 3. Model for the electron transfer reactions between amicyanin and cytochrome c-551i. The shapes represent methylamine dehydrogenase (M), amicyanin (A), and cytochrome c-551i (C). Black and lined patches represent, respectively, hydrophobic and hydrophilic surface domains on amicyanin and cytochrome c-551i. The most direct access to the copper and heme cofactors is via these hydrophobic domains on each protein.