Molecular Crystals and Liquid Crystals
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gmcl18

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Version of record first published: 04 Oct 2006.

To cite this article: Ling Qin , Karla K. Rodgers & Stephen G. Sligar (1991): Electron Transfer between Cytochrome B^5 Surface Mutants and Cytochrome C, Molecular Crystals and Liquid Crystals, 194:1, 311-316

To link to this article: http://dx.doi.org/10.1080/00268949108041181

Mol. Cryst. Liq. Cryst., 1991, vol. 194, pp. 311–316
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Printed in the United States of America

ELECTRON TRANSFER BETWEEN CYTOCHROME B_5 SURFACE MUTANTS AND CYTOCHROME C

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Abstract The surface amino acid residues of rat liver Cytochrome b_5 (Cyt b_5) thought to be involved in forming salt bridges with horse heart Cytochrome c (Cyt c) have been mutated in order to probe the relation between the protein complex structure and the electron transfer function. The intracomplex electron transfer rate constants measured by flash photolysis, in aqueous solution at pH 7.3, range from about 2000 - 5000 s^{-1}. There is no significant difference between the wild type (WT) and the mutant Cyt b_5's in the electron transfer rate constant. The electron transfer rate constants are also measured under different pH and solvent conditions. These experiments indicate that the protein dynamic fluctuations may play important roles in the search of favorable conformations in order for electron transfer to occur.

Keywords: Cytrochrome b_5, cytochrome c, mutagenesis, electron transfer

INTRODUCTION

Cyt b_5 and Cyt c have been the subject of intensive study because they represent a good model system for understanding protein-protein interaction whereby the electrostatic interactions play a major role. Various techniques have been used to study the interaction between Cyt b_5 and Cyt c. Difference[1] and fluorescence[2] spectroscopies were used to study the affinity of the complex. Computer modelling,[3] molecular dynamics,[4] electrostatic calculation,[5] NMR[6] and high pressure[7] spectroscopies were employed to map the interaction domain in the complex. It is now generally accepted that several lysine residues (K) of Cyt c and glutamate (E) and aspartate (D) residues of Cyt b_5 are involved in forming salt bridges in the complex. In a previous study[7] we mutated the residues E48 and D60 of Cyt b_5, thought to be involved in forming salt bridges with Cyt c, to glutamine (Q) and asparagine (N), respectively. Binding studies indeed show that these residues reside at the interface of the

complex as the mutant [Cyt b_5/Cyt c] complexes have larger K_d's (Table 1). Since the complexes formed between mutant-Cyt b_5 and Cyt c bind less tightly, the recognition between them should be less specific. In this study, we examined the electron transfer (ET) kinetics between Cyt b_5 and Cyt c in order to see if the change in affinity and specificity have any significant effect on the electron transfer in the complex.

RESULTS AND DISCUSSION

Laser flash photolysis has been used to study electron transfer kinetics. The solution saturated with argon included 0.5 mM EDTA and about 100 μM acridine. Presumably the excited state of acridine acts as primary electron donor. The second order rate constants for reduction of Cyt c and Cyt b_5 by acridine are $8.7x10^8$ $M^{-1}s^{-1}$ and $1.8x10^9$ $M^{-1}s^{-1}$, respectively. The reduction of Cyt b_5 also consists of a 10% slower process, the reason for which is not clear at the moment. We also measured the reduction potentials (E_m) of the Cyt b_5 proteins for reference purposes (Table 1).

TABLE 1 Experimental Data for Cyt b_5 at pH 7.3

Cyt b_5	E_m(mV)	K_d[a)](μM)	k(s^{-1})
WT	-1	0.4	$1.7x10^3$
E48Q	+7	0.8	$4.4x10^3$
D60N	+9	1.00	$3.7x10^3$
D66S[b)]	+3	0.41	$3.0x10^3$
WT(pH 8)		1.47	$3.3x10^3$

a) At 2 mM ionic strength.

b) D66, which does not reside at the complex interface, was changed to S66 as a control.

The Cyt b_5 and Cyt c form a 1:1 complex at the low ionic strength as is evidenced by binding studies. The observed ET rate constants are intracomplex because they are independent of protein concentrations (7 μM to 20 μM each of Cyt b_5 and Cyt c). The reduction of Cyt b_5 and its subsequent electron transfer to Cyt c were monitored at 556 nm and at 428 nm (Figure 1) and the formation of reduced Cyt c at 545 nm (Figure 2).

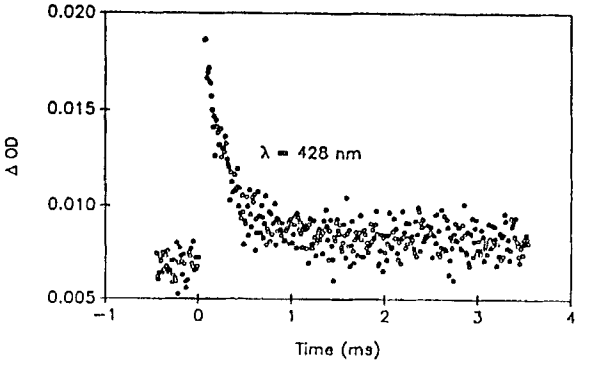

FIGURE 1 Decay of reduced E48Q Cyt b_5 mutant due to ET to oxidized Cyt c in the complex.

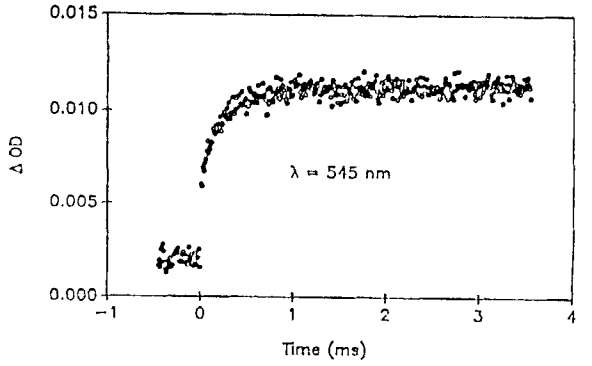

FIGURE 2 Growth of reduced Cyt c in the [E48Q Cyt b_5/Cyt c] complex.

Two processes were observed for the reduction of Cyt c, the faster one is due to the direct reduction by acridine and the slower one, which is compatible to the decay of reduced Cyt b_5, is due to the intracomplex ET from Cyt b_5. Table 1 lists the observed first order ET rate constants for the WT and mutant Cyt b_5's.

The rate constant for WT Cyt b_5 is comparable to that reported by Miller and Mclenden.[8] No significant difference was found between the WT and the mutant Cyt b_5's in the ET rate constants observed at pH 7.3. We also measured the ET rate constant for the WT Cyt b_5 at pH 8.0. Mauk[5] initially proposed that, at pH 8.0, 40% of the complexes have the heme Fe to Fe distance 8 Å larger (18 to 26 Å) than that at pH 7.3. This model was, however, primarily based on an electrostatic calculation[5] with an erroneous Cyt b_5 amino acid sequence.[6] According to this model one would expect a large decrease of ET rate compared to that at pH 7.3. This is not supported by the present study.

The above experiments show that the binding specificity, somewhat disrupted by the mutagenesis, does not appear to change the ET rate constants in a significant way. This is consistent with the increasingly accepted view that the complementary electrostatic interactions that stabilize and orient the reaction partners in the complex are quite flexible. These associations differ from enzyme-substrate complexes that typically incorporate very specific interactions to more rigidly fix favorable intermolecular orientation. The relative lack of recognition specificity observed between electron transfer proteins allows many nonphysiological reaction partners, like Cyt b_5 and Cyt c, to react as fast as true physiological partners, provided that appropriate surface charge distributions that are required for molecular orientation are preserved. This relative lack of specificity is in agreement with the fact that the ET is generally not a rate limiting step in metabolic pathways. A recent NMR study[6] indicates that at least 6 lysine residues of Cyt c are involved in complexation with Cyt b_5, in contrast to Salemme's proposal[3] that only 4 salt bridges are involved in the complex. These results can be rationalized by the existence of an ensemble of [Cyt b_5/Cyt c] complexes or local movement about the specific regions of their respective complementarily charged surface. In order to test that the flexibility is important for the electron transfer function, we measured the ET rate constant for the native complex in more viscous solvents in the hope that the solvent will hinder the flexibility or slow down dynamic fluctuations in the complex. Figure 3 shows the observed rate constants as a function of glycerol concentration at pH 7.4. This decrease of rate at higher glycerol concentration is not due to the hindered translational diffusion because the observed rate constants do not change at the protein concentrations used, as is shown in Figure 4.

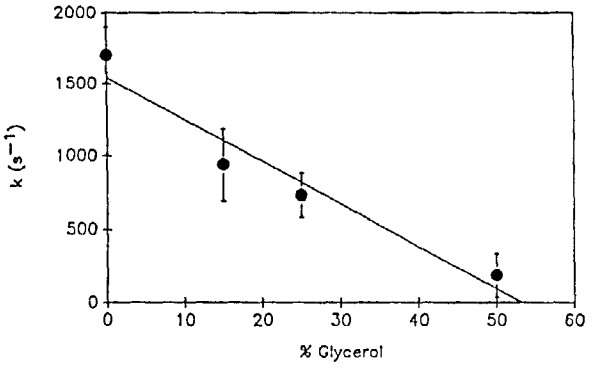

FIGURE 3 The observed ET rate constants as a function of glycerol concentration at pH 7.4.

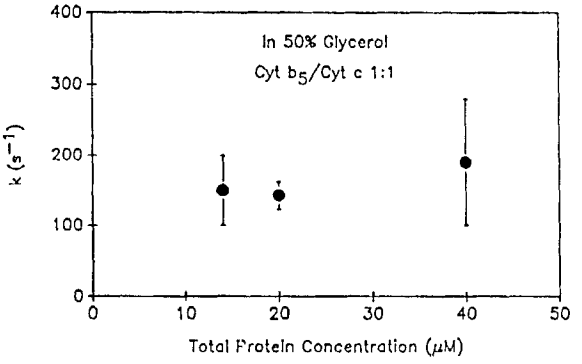

FIGURE 4 The observed ET rate constants as a function of protein concentration in 50% glycerol solution.

The binding study, depicted in Figure 5, shows that the glycerol does not change the thermodynamic equilibrium of the complex and that they still bind in a 1:1 stoichiometry and at similar strength compared with that in aqueous solution.

Hoffman[9] observed a similar rate decrease in ethylene glycol mixed solvents for the [Zn-Cyt c peroxidase/Cyt c] complex and Kostić[10] observed no ET in the crosslinked [Cyt c/plastocyanin] complex. These observations imply that reorientation or dynamic fluctuations may be important for efficient

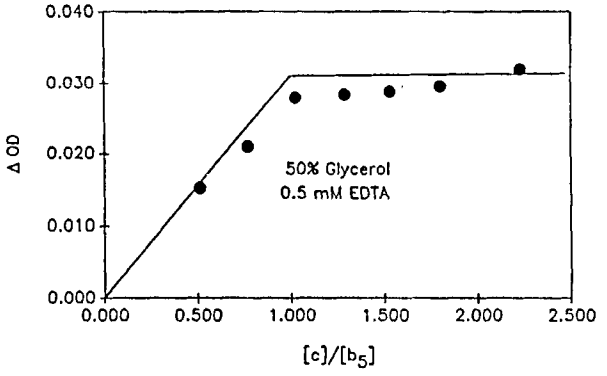

FIGURE 5 The binding of WT Cyt b_5 with Cyt c in 50% glycerol.

ET to occur. However, one can not rule out other explanations. For example, proteins may have different dockings in different solvents and the solvent relaxation may play dominant roles in certain ET processes. Further experiments are under way to probe these possibilities.

This research was supported by NIH Grant GM31756.

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