Appendix

INTRODUCTION

The ligand substitutions that occur during folding of horse heart ferrocytochrome *c* were investigated in our laboratory using transient absorption spectroscopy.[1] Following the kinetics in the Soret absorbance region, the rate of (Met80)(His18)Fe(II)cyt *c* formation was found to decrease with increasing imidazole (Im) concentration; at very high [Im] (>100 mM) there was no sign of (Met80)(His18)Fe(II)cyt c_f formation. However, it was suggested, that even in presence of high [Im], the protein can still fold but perhaps around the misligated heme. The optical changes associated with this folding could not be detected by transient absorption spectroscopy because the Soret absorption region reports mainly on the heme ligation state and small backbone conformational changes most likely will not be detectable. To determine if the backbone conformational changes do occur, folding of DNS(C102)-cyt c^{II} was carried out in presence of imidazole with NADH as photochemical sensitizer. The change in DNS fluorescence intensity was followed as a function of time. The experimental findings from these studies are described in this chapter.

The transient-fluorescence kinetics employing a reduced form of nicotinamide adenine dinucleotide (NADH) as a photochemical sensitizer[2] indicated that some very fast conformational changes occur on the timescale of about 300 μs. However, electron injection with NADH as a photochemical sensitizer is somewhat slow (about 100 μs), excitation of NADH creates background fluorescence and the overall electron transfer process is not reversible. We took advantage of the $Ru(bpy)_3^{2+}$ photochemistry to inject electrons into oxidized cyt *c*. Gary A. Mines et al.[3] had shown that reductive quenching of

$Ru(bpy)_3^{2+}$excited state with p-methoxy-N,N'-dimethylaniline (MeODMA) generates enough $Ru(bpy)_3^{+}$ ($E°(Ru^{2+}/Ru^{+})$ = -1.28 V vs. NHE[4]) to give fast (about 10 μs), high yield electron injection into oxidized cyt *c*. We studied an electron injection into unfolded DNS(C102)-cyt c^{III} by time-resolved transient absorbance. A promising reductive flash-quench-scavenge system for a delivery of reductive pulse to generate DNS(C102)-cyt c^{II} was established.

MATERIALS AND METHODS

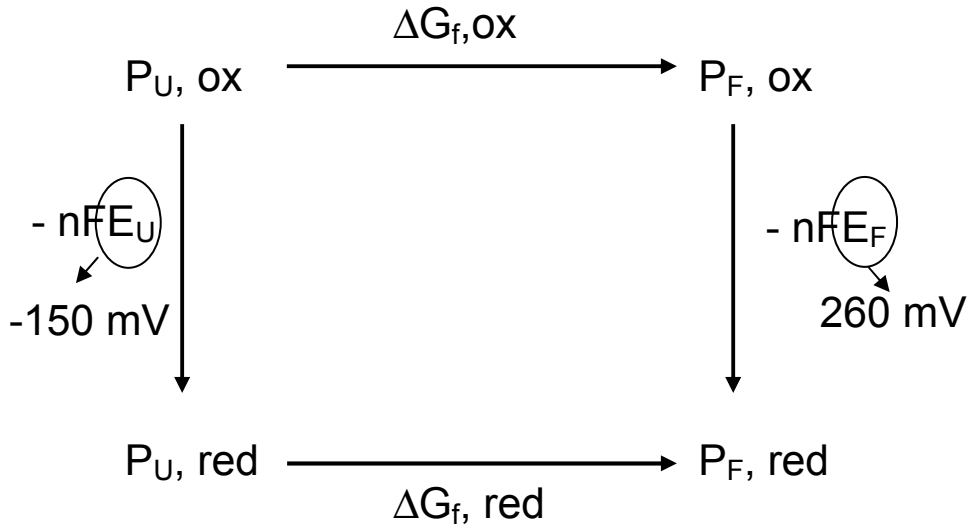
The materials and methods relevant to this chapter are described in the corresponding sections of Chapter 2 and Chapter 3. Those introduced in this chapter are described below.

General

Ascorbic acid was from Sigma. *p*-methoxy-N,N-dimethylaniline (MeODMA) was a gift of Ivan Dmochowski.

Electron-Transfer Triggered Folding/Unfolding

A thermodynamic cycle connecting oxidized (P_{ox}) and reduced (P_{red}) forms of a protein in its folded and unfolded states is shown in **Figure A.1**. If potentials for folded and unfolded states are different, then the free energies for folding for oxidized and reduced forms of protein will also differ



$\Delta\Delta G_{f,\ red\text{-}ox} = -nF(\Delta E_{F-U}) = 410mV\ (410meV) = 10\ kcal/mol$

Figure A.1. Thermodynamic cycle showing a relationship between folding free energies (ΔG_f,ox) and thermodynamic potentials (E) in a redox protein (P). n is the number of electrons transferred and F is the Faraday constant. The subscripts refer to the states of protein: U = unfolded, F = folded, ox = oxidized, red = reduced.

($\Delta\Delta G_{folding}$ (red-ox) = -nF(ΔE_{F-U})). In aqueous solution $\Delta\Delta G_f$ (H_2O)(red-ox) might be as large as 10 kcal/mol (the reduced form has a more favorable free energy for folding than the oxidized form).

Under denaturing conditions, according to Eq. 2.12, Chapter 2, the unfolding midpoints occur at denaturant concentrations ($\Delta G_f(H_2O)_{ox}/m_{D,ox}$) and ($\Delta G_f(H_2O)_{red}/m_{D,red}$) for oxidized and reduced forms of the protein, respectively. If $m_{D,ox} \approx m_{D,red}$, the positions for these midpoints will occur at quite different denaturant concentrations. Indeed a range of denaturant concentrations can be found where one of the forms is 99% unfolded while

the other form is 99% folded. Hence electron injection into the heme/electron removal from the heme will initiate folding/unfolding (unfolding curve of DNS(C102)-cyt *c*, **Figure 2.25, Chapter 2**).

Folding DNS(C102)-cyt *c* in Presence of Imidazole, Followed by Fluorescence

Samples typically contained 50 μM protein, 100 μM NADH, 0 to 400 mM imidazole and [GuHCl] = 1.15 - 1.4 M (NaPi buffer, μ = 0.1, pH 7). They were Ar-degassed with repeated pump/fill cycles on a Schlenk line in vacuum cells fitted with 1 mm quartz cuvette sidearms. Samples were excited by 308 nm light from XeCl excimer laser (Lambda Physik LPX201i). The DNS fluorophore was excited by HeCd 325 nm laser and luminescence decay was detected at 500 nm. Experimental data were fit to first order kinetics using MATLAB. Some chromophore bleaching by the probe laser was detected and kinetics were corrected for it. Uncertainties in estimated rate constants were within 20%.

Transient Difference Absorption Spectroscopy

Difference spectroscopy is commonly used when it is necessary to measure precisely very small spectral changes in the system. The difference spectra can be acquired as a function of time allowing one to study kinetics.

In the light absorption measurement the fraction of light absorbed ($-dI/I$) is simply proportional to the number of absorbing molecules:

$$-dI/I = C\varepsilon dl \qquad \text{(A.1)}$$

where C is concentration of the absorbing molecules; ε is a proportionality constant called molar extinction coefficient and dl is thickness of the sample (assumed to be sufficiently small so that the light intensity is constant as light passes through the sample). Integration of equation (A.1) from the initial (I_0) to the final intensity (I) throughout the whole sample and conversion to log base 10 gives

$$log(I_0/I) = C\varepsilon' dl \qquad \text{(Beer-Lambert law)}$$

$\varepsilon' = \varepsilon * 2.303$

To measure the difference in light absorption an instrument has to detect

$$\log (I_B/I_S) = A_S - A_B$$

$\log I_S = \log(I_0) - A_S$ is the intensity of the light that comes out after passing through the "sample".

$\log I_B = \log(I_0) - A_B$ is the intensity of the light that comes out after passing through the "blank".

In all experiments described in this chapter, the "sample" refers to a solution that has been excited with a laser pulse. The "blank" refers to exactly the same solution as the "sample" except unexcited (no laser pulse). Thus the method measures difference in absorbance by molecules in the ground and the excited states. The *transient difference absorbance spectrum* will represent the absorption features of the transient population of the excited species.

Transient absorption kinetics were monitored at selected wavelengths, after excitation of DNS(C102)-cyt c^{III} ([DNS(C102)-cyt c^{III}] = 36 μM was a typical concentration), 10μM $Ru(bpy)^{2+}$, 10 mM MeODMA (all in about 2.8 M GuHCl, pH 7.0, 20°C). Samples were Ar-degassed by repetitive pump/fill cycles in vacuum cells fitted with 1 mm quartz

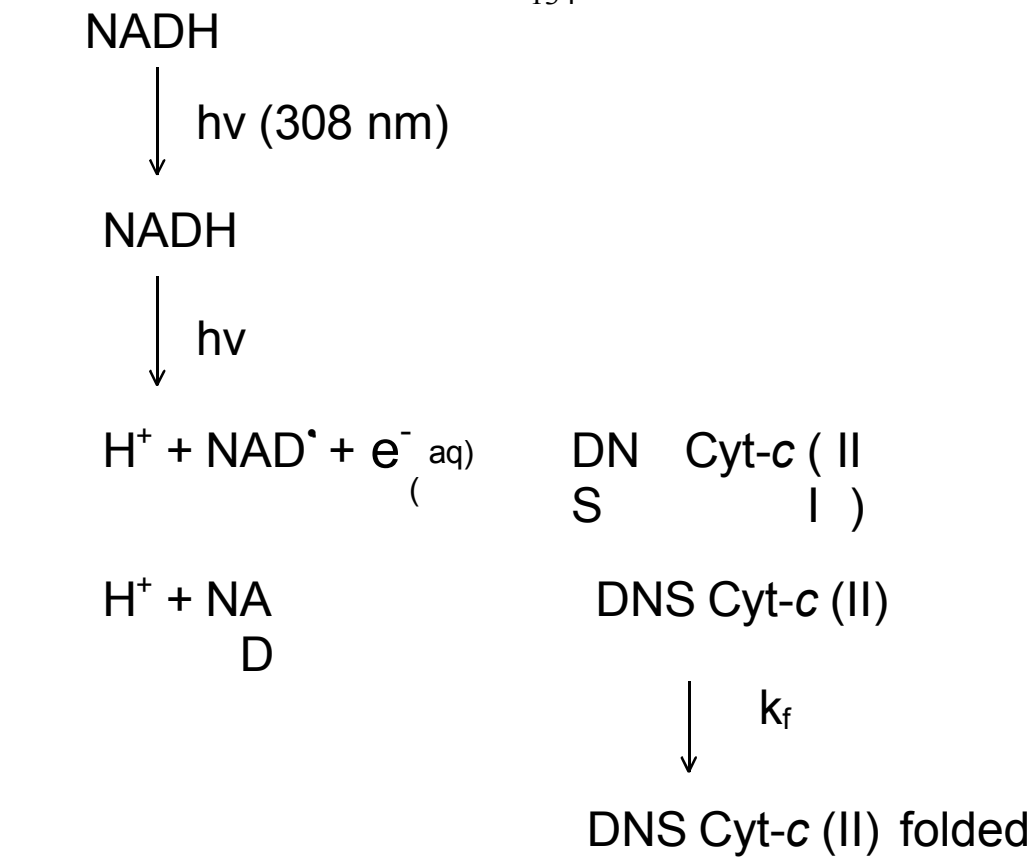
cuvette side arms. Samples that contained ascorbic acid (10 mM) were prepared in the dark and kept on ice until laser experiments. The excitation source was a dye laser (Lambda Physik FL3002; Coumarin 480, 20 ns/pulse, 480 nm, 1-5 mJ/pulse) pumped by a XeCl excimer laser (Lambda Physik LPX210I). Single-wavelength transient absorbance traces were collected by directing light from a 75 W xenon arc lamp through the sample, colinearly with the excitation beam, into a monochromator. The signal was detected with a Hamamatsu R928 photomultiplier tube. It was amplified using a DSP 1402E programmable amplifier, and digitized with a Tektronix R710 200-MHz 10-bit transient digitizer connected to a computer. All the transient-absorbance kinetic traces were fit to single or biexponential functions using the KINFIT program (written by Dr. Jay R. Winkler) or Origin software.

RESULTS AND DISCUSSION

Folding DNS(C102)-cyt *c* in Presence of Imidazole Followed by Fluorescence

NADH was employed as a sensitizer for injecting electrons into unfolded oxidized protein. Two-photon 308 nm excitation generates two powerful reductants $NAD^{\bullet}$ and a solvated electron (**Figure A.2**). Both reductants inject electrons into unfolded protein in about 100 μs.

The imidazole slows the rate of binding of Met80 to the heme in the reduced protein. At very high [Im] (> 100 mM) no sign of (Met80)(His18)Fe(II)cyt *c* was observed even after 5 s[1,2]. In this work, folding was followed by a change in DNS fluorescence intensity and an interesting dependence of folding rates on imidazole concentration was noticed. As can be seen in Table A.1, the rate that coincides with Met80 ligation[2]



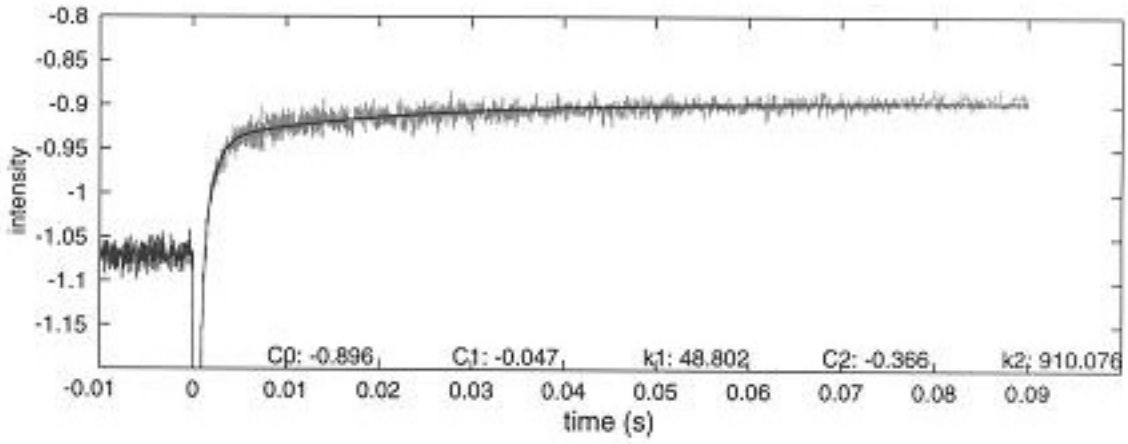


Figure A.2. Top: scheme of photoinduced reduction of DNS(C102)-cyt c^{III} with NADH as photochemical sensitizer; bottom: transient fluorescence kinetics of DNS(C102)-cyt *c* (50 μM, 100 μM NADH, 50 mM imidazole, 1.16 M GuHCl, NaPi, pH 7.0, μ 0.1, $\lambda_{DNS,\ ex}$ = 325 nm, $\lambda_{NADH,\ ex}$ = 308 nm, λ_{obsd} = 500 nm). The smooth line is the best fit. k_1 = 48.8 s^{-1} is the rate attributed to conformational changes in protein.

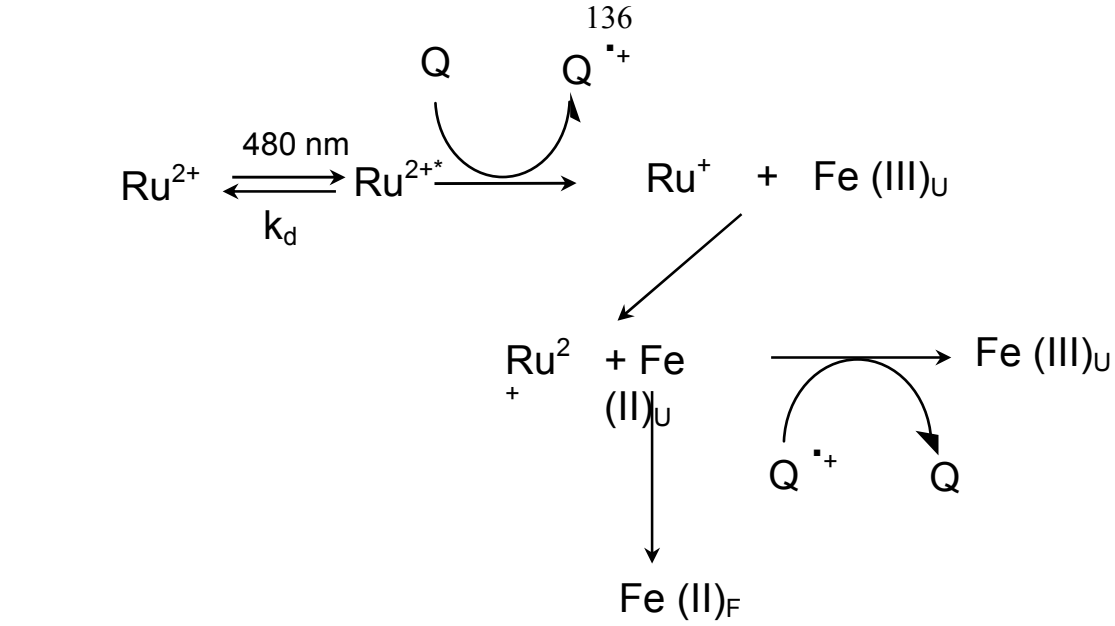
(about 40 s^{-1}, 1.15-1.4 M GuHCl, pH 7, no imidazole) slows down to about 0.5 s^{-1} in presence of very high [Im] (400 mM). Since even at very high [Im], some conformational changes are observed by fluorescence, the protein probably is able to fold, but perhaps around a misligated heme.

Table A.1. Dependence of DNS(C102)-cyt *c* folding rates on imidazole concentration.

Imidazole Concentration (mM)	k_f (s^{-1})
0	40
20	37
50	45
100	2.5
400	0.5

Reductive Flash-Quench-Scavenge Experiments

Mines et al.[3] showed that reductive quenching of $Ru(bpy)_3^{2+}$* with *p*-methoxy-N,N'-dimethylaniline (MeODMA) ($k_q = 6 \times 10^8$ M^{-1} s^{-1}, [5]) generates $Ru(bpy)_3^{+}$ (Ru^{+} represents species in which ruthenium is in 2+ oxidation state and one of the ligands is a radical anion**,** yield of $Ru(bpy)_3^{+}$ is 0.58,[6] $E°(Ru^{2+}/Ru^{+})$ = -1.28 V vs. NHE[4]) that gives fast, high yield electron injection into unfolded oxidized cytochrome *c*. The reductive quenching scheme is shown in **Figure A.3** (top) where Q = MeODMA. On the bottom of **Figure A.3** the kinetic trace for the decay of $Ru(bpy)_3^{2+}$* fluorescence is shown. The sample was excited with 480 nm light. $k_{obs} = 2.8 \times 10^7$ s^{-1} (τ = 34 ns) is a pseudo-first-order rate constant described by $k_{obs} = k_0 + k_q [Q]$; where k_0^{-1} is the lifetime of $Ru(bpy)_3^{2+}$*



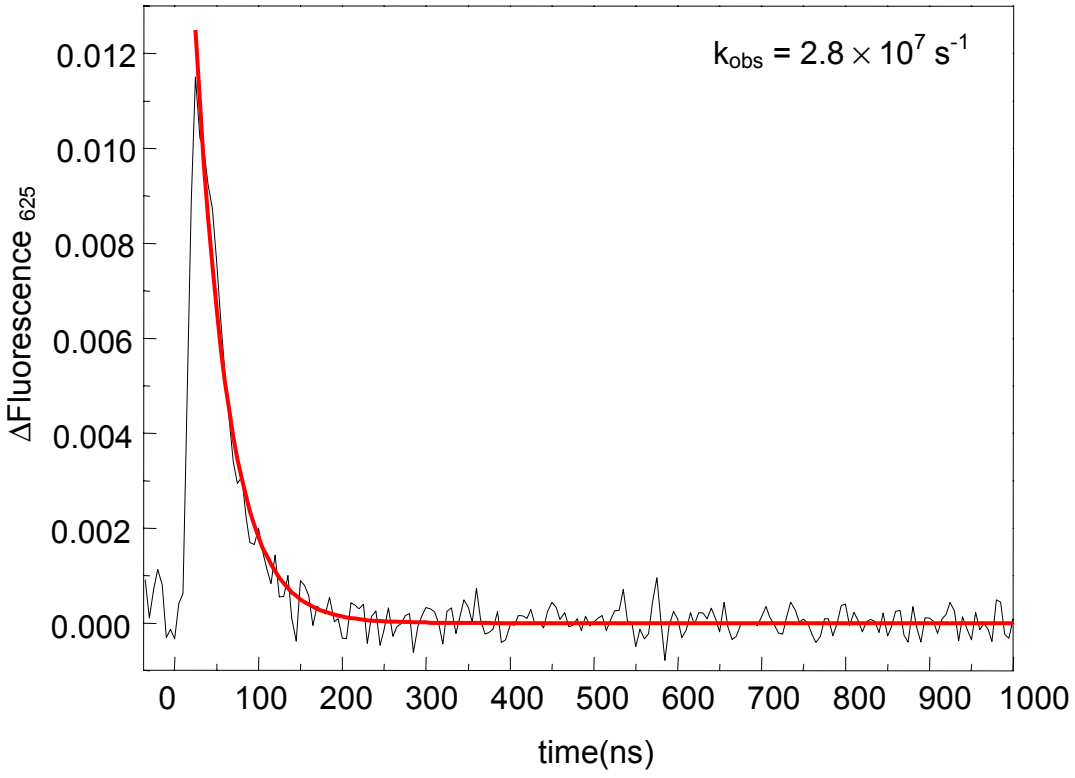


Figure A.3. Top: reductive quenching scheme; Q = MeODMA, Fe = heme in DNS(C102)-cyt *c,* Ru = ruthenium center in $Ru(bpy)_3$; bottom: kinetic trace for the decay of $Ru(bpy)_3^{2+*}$ triplet excited state in presence of Q.

when no quencher is present (about 600 ns in aqueous solution); Q = MeODMA. **Figure A.4** shows kinetic traces obtained upon photoinduced reduction ($k_{red} = 1.9 \times 10^5$ s^{-1}) of unfolded DNS(C102)-cyt c^{III}. On the bottom of **Figure A.4** reoxidation of heme by $MeODMA^{\bullet+}$ is seen to occur on the timescales of approximately 200 μs (the reoxidation is a second-order reaction and is not accurately described by the first-order biexponential fit shown in **Figure A.4** bottom). In order to eliminate the possibility of involvement of the dissolved oxygen in facilitating this unexpectedly fast heme reoxidation, similar photoinduced reduction experiments were performed on a poorly degassed sample and the transient absorption kinetic trace was collected at 420 nm (data not shown). The yield of reduction was much smaller and the heme reoxidation was noticeably faster (occured in ~ 25 μs) compare to the one observed in **Figure A.4.**

To slow down heme reoxidation, reductive flash-quench-scavenge experiments were performed. **Figure A.5** (top) shows reductive flash-quench-scavenge scheme where As stands for ascorbic acid used to scavenge $MeODMA^{\bullet+}$. The heme reoxidation was indeed much slower when ascorbic acid was added (**Figure A.5**, bottom). No reoxidation was observed on timescales of up to 500 μs. The electron injection rate slowed down slightly but was still fast ($k_{red} = 6.13 \times 10^4$ s^{-1}). The entire process was reversible and complete. Heme reoxidation occured within 40 ms (**Figure A.6**). Transient absorption kinetic traces were acquired at two other wavelengths that were also sensitive to the change in oxidation state of the heme (400 nm and 550 nm). The transient absorption kinetic trace acquired at 550 nm is shown in **Figure A.6** (bottom). At the end of reductive flash-quench-scavenge experiments some accumulation of the reduced protein was observed which was

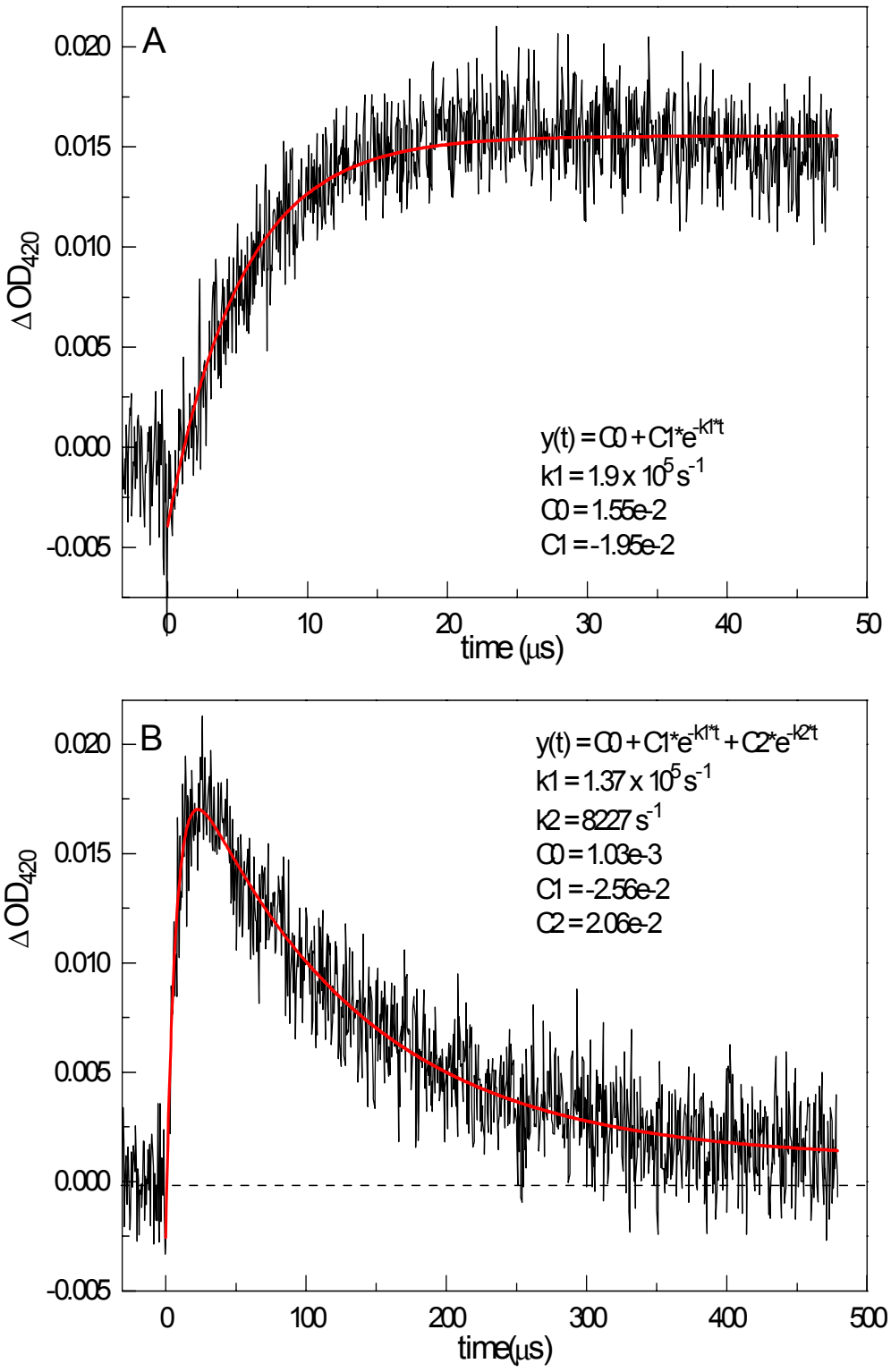


Figure A.4. Transient absorption kinetics monitored at 420 nm after excitation (λ_{ex} = 480 nm) of 36 μM DNS(C102)-cyt *c*, 10 μM $Ru(bpy)_3^{2+}$, 10 mM MeODMA (all in ~2.8 M GuHCl, NaPi (μ = 0.1, pH 7, 20°C). The lines are the best fits to single exponential (top) and biexponential (bottom) functions.

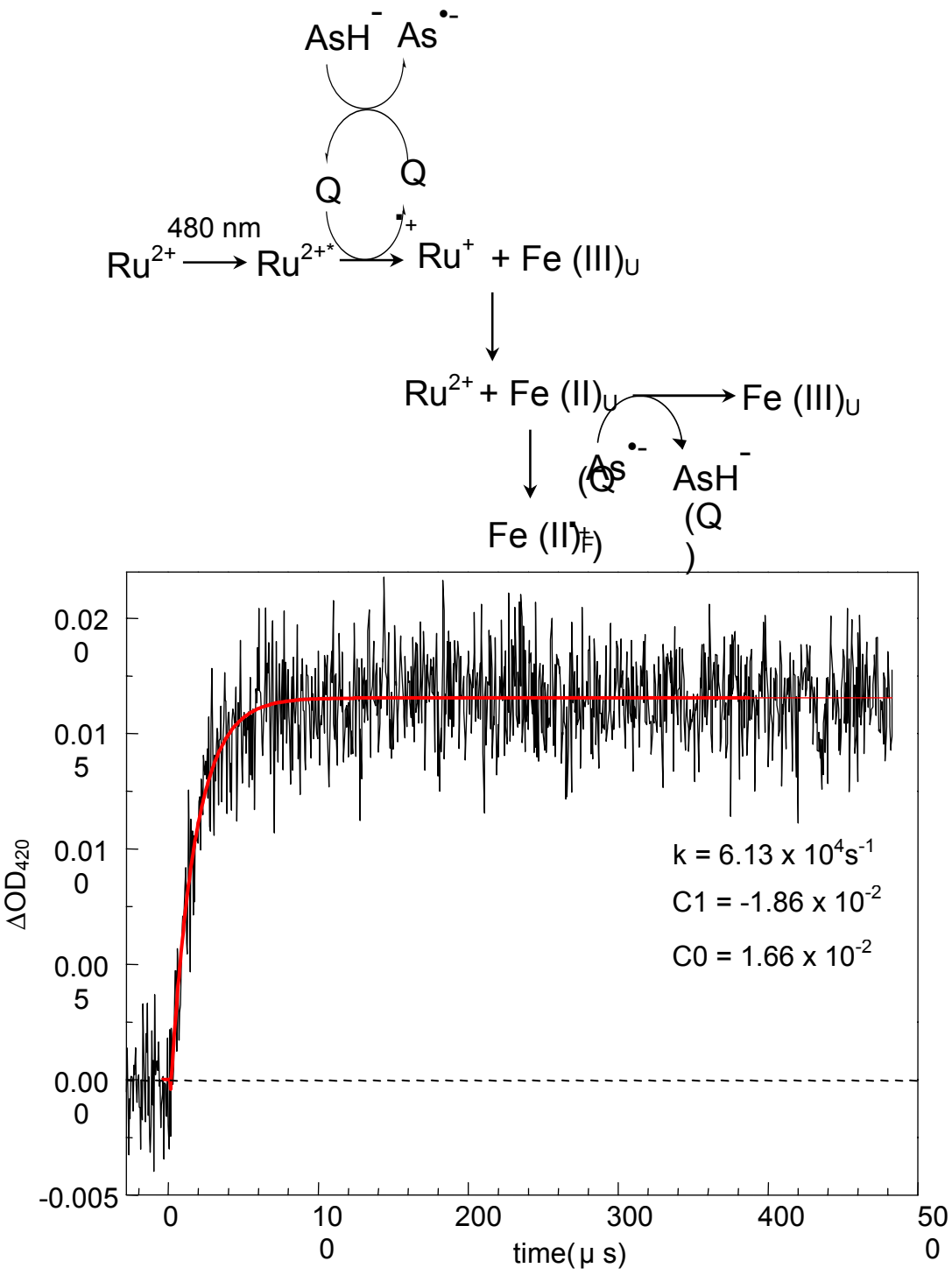


Figure A.5. Photoinduced reduction of DNS(C102)-cyt c^{III}; top: scheme, Q=MeODMA; As = ascorbic acid, Fe=heme in DNS(C102)-cyt *c,* Ru = ruthenium center in $Ru(bpy)_3$; bottom: transient absorption kinetics (λ_{obs} = 420 nm) observed after excitation of 36 μM DNS(102)-cyt *c*, 10mM $Ru(bpy)_3^{2+}$, 10 mM MeODMA, 10 mM ascorbic acid (all in ~2.8 M GuHCl, pH 7.0, 20°C). The line is the best fit to a single exponential function. λ_{ex} = 480 nm. Total number of shots: 500.

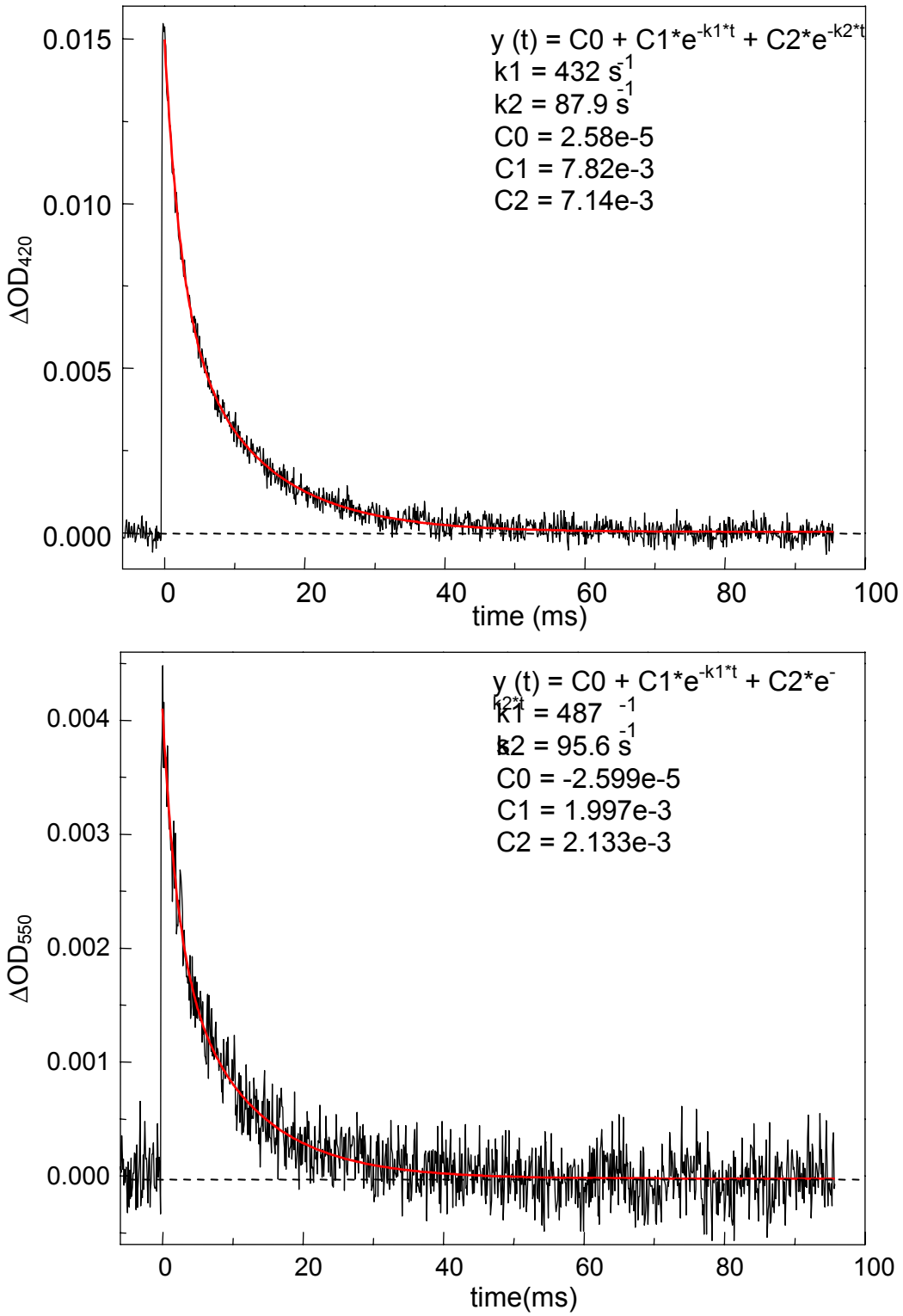


Figure A.6. Reoxidation of DNS(C102)-cyt c^{III}; top: transient absorption kinetics (λ_{obs}=420 nm); bottom: transient absorption kinetics (λ_{obs}=550 nm). Experimental conditions are as in Figure 5.5.The lines are the best fits to a biexponential function.

most likely due to a slow reduction (6.6×10^3 M^{-1} s^{-1}) of DNS(C102)-cyt c^{III} by perhaps a transiently formed ascorbyl radical ($E_{m,7}$ ($As/As^{\bullet-}$) = -210 mV vs NHE, where As is dehydroascorbate and $As^{\bullet-}$ is ascorbyl radical).

One of the implications of using ascorbate as a scavenger is that at low GuHCl concentration (2 M and lower) ascorbic acid will reduce DNS(C102)-cyt c^{III} ($E_{m,7}$ (As/AsH^-) = 60 mV vs. NHE, where As is dehydroascorbate and AsH^- is ascorbate monoanion formed at pH 7 upon ascorbic acid dissociation; pK_{a1} = 4.25; pK_{a2} = 11.79).

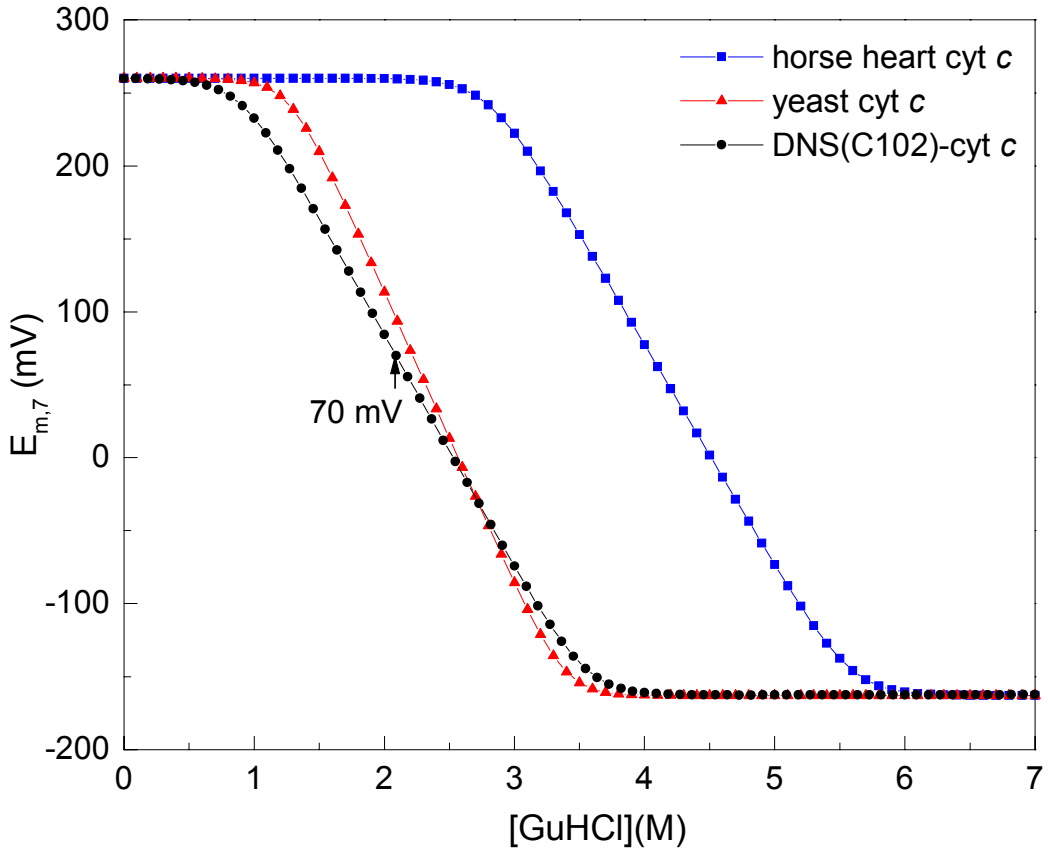


Figure A.7. $E_{m,7}$ as a function of [GuHCl] for DNS(C102)-cyt *c*, horse heart cyt *c* and yeast iso-1 cyt *c*.

The plot of $E_{m,7}$ versus [GuHCl] for DNS(C102)-cyt *c*, horse heart and yeast cyt *c* (**Figure A.7**) was generated using Equation A.2 with experimentally determined K_F^{III} (DNS(C102)-cyt *c*, thermodynamic data **Chapter 2**; horse heart and yeast iso-1 cyt *c*, thermodynamic data Ref. 7) and literature values of $E(Fe_U^{III}/Fe_U^{II})$ and $E(Fe_F^{III}/Fe_F^{II})$.

$$E_h = E_{m,7} + \frac{0.06}{n}\log\frac{[ox]}{[red]}$$

where E_h is a redox potential with respect to standard hydrogen electrode; $E_{m,7}$ is the midpoint redox potential (when $[ox] = [red] \neq$ standard state at pH 7.0); 0.06 = 2.303RT/F (at 25°C)

oxidized state: $Fe_U^{III} \rightarrow Fe_F^{III}$ $\quad K_F^{III} = \frac{Fe_F^{III}}{Fe_U^{III}}$ $\quad Fe_U^{III} = \frac{Fe_F^{III}}{K_F^{III}}$

reduced state: $Fe_U^{II} \rightarrow Fe_F^{II}$ $\quad K_F^{II} = \frac{Fe_F^{II}}{Fe_U^{II}}$ $\quad Fe_U^{II} = \frac{Fe_F^{II}}{K_F^{II}}$

oxidized state total: $Fe_U^{III} + Fe_F^{III} = [ox]_t$

reduced state total: $Fe_U^{II} + Fe_F^{II} = [red]_t$

$$E_h = E_{m,7} + 0.06\log\frac{[ox]_t}{[red]_t} = E_{m,7} + 0.06\log\left[\frac{Fe_F^{III} + \frac{Fe_F^{III}}{K_F^{III}}}{Fe_F^{II} + \frac{Fe_F^{II}}{K_F^{II}}}\right]$$

$$E_h = E_{m,7} + 0.06\log\frac{Fe_F^{III}}{Fe_F^{II}}\frac{(1+K_F^{II})}{(1+K_F^{III})}$$

if $[Fe_F^{III}] = [Fe_F^{II}]$ then $E_h = E(Fe_F^{III}/Fe_F^{II}) = 0.26$ V

$$E_{m,7} = E(Fe_F^{III}/Fe_F^{II}) - 0.06\log\left[\frac{(1+\frac{1}{K_F^{III}})}{(1+\frac{1}{K_F^{II}})}\right]$$

$$E_{m,7} = 0.26V - 0.06V \log\left[\frac{(1+K_F^{III})K_F^{II}}{(1+K_F^{II})K_F^{III}}\right] \quad \text{(A.2)}$$

due to experimental difficulty in determination of K_F^{II} it can be substituted by expression (A.3).

$$0 = -nFE(Fe_U^{III}/Fe_U^{II}) + \Delta G_F^{II} - (-nFE(Fe_F^{III}/Fe_F^{II}) - \Delta G_F^{III}$$

$$\Delta G_F^{II} = nFE(Fe_U^{III}/Fe_U^{II}) - nFE(Fe_F^{III}/Fe_F^{II}) + \Delta G_F^{III} = -RTlnK_F^{II}$$

$$K_F^{II} = \exp\left(\frac{-0.26V + (-0.15V) + \Delta G_F^{III}}{-RT}\right) \quad \text{(A.3)}$$

A number of other potential scavengers for $MeODMA^{\bullet+}$ should be investigated. Those include hydroquinone, catechol and $Ru(NH_3)_6^{2+}$.

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