Function and Characterization of cyt c552 of *Vibrio Cholerae*

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Abstract

The thesis focuses on characterization of cytochrome c552. The physical chemical properties, and especially midpoint potential of a cytchrome puts limits on the physiological role (but does not determine it): to function effectively in an electron transfer chain. Theoretically, the midpoint potential (Em) should not be much more negative than the physiological reductant or much more positive than its oxidant. The spectrum c552 in the oxidized and reduced form have been determined and put on an absolute scale. The absorption maximum at the alpha peak is 551.3 nm. The midpoint potential was determined by equilibration with the redox indicator dye Tetral Methyl Phenylene Diamine (TMPD). The midpoint potential was relatively independent of pH over the range of pH 6.5 to pH 8.5 with the value around 200 mV, significantly lower than mitochondrial cytochrome c (257 mV). This suggests the role of c552 is not to function with the terminal oxidase.

Background

Cytochrome c552 of vibrio cholerae

The genome of *vibrio cholerae* contains one open reading frame coding for a protein homologous to cytochrome c552 of *Pseudomonas Nautica*. The sequence is available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=75817024&dopt=GenPept. In the vibrio genome this protein has been annotated as cytochrome c553, however, we call it c552 after the *P. Nautica* homolog (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=5822017&dopt=GenPept). Li-Shar Huang purified some soluble cytochromes from *vibrio cholerae*. One of them was crystallized and the structure was solved by molecular replacement using the *P. Nautica* structure. The sequence proved to be that of *vibrio cholerae* c553. The project of this thesis is biochemical characterization of this protein which we call c552.

Redox Chemistry

In definition, midpoint potential is if a redox reaction could be coupled to a electrode, more strongly reducing reaction, would make the electrode more negative by transferring electron to it. Therefore, the electrode is a measure of reducing power, with more negative potential implying greater ability to transfer electrons. I.e. stronger reducing power. Reducing power is determined not only by chemical factors (how willing the compound is willing to give up a electron), but also by the ratio of reduced/oxidized form of the compound. If most of the compound is reduced, and only a small amount is oxidized, there will be a greater chance to reduce something than to oxidize something. Midpoint potential is the electric potential on the electrode where the redox couple will be half reduced at equilibrium. A more negative midpoint potential, therefore implies a stronger reducing agent, harder to be reduced, easier to oxidize, hence a higher tendency to give up electron.

In order to put reducing power on an absolute scale, the reducing power of various compound is compared to that of (H2/2H+). Because this is often measured using electrodes and

voltmeter, reducing power is measured in milivolts. The Nernst equation tells us that a 59 mV difference in midpoint potential corresponds to a 10 fold difference in equilibrium constant.

$$E_h = E^o - (2.303\ RT/nF) \log ([R]/[O])$$

Substituting for the constants (2.303 RT/nF):

$$E_h = E^o - (59) \log ([R]/[O]) \qquad \text{(in mV at 278 K for n=1)}$$

Where E_h is the potential measured (ambient redox potential) E^o is the midpoint potential (standard reduction potential). [O] and [R] are the moalr concentrations of the oxidized and reduced forms, respectively. Therefore, if E_h of a solution is measured, and the ratio of reduced and oxidized forms is known, one can determine the midpoint potential. The concentration ratio of reduced and oxidized forms can be readily determined from absorption spectroscopy.

Alternatively, instead of using voltmeter and electrode, another way to measure the midpoint potential (reducing power) of a redox agent is to measure its redox equilibrium with another redox agent of known midpoint potential. If the two redox reagents are in equilibrium, they are at the same ambient redox potential E_h. The second redox reagent serves as a redox indictator dye. In the experiment described here, absorption spectroscopy is used to measure the oxidized/reduced ratio for both the cytochrome c552 and a redox dye of known midpoint potential. The equilibrium constant is calculated:

$$Keq = [Cyt_{ox}][Dye_{red}]/[Cyt_{red}][Dye_{ox}]$$

Applying the Nernst equation (see above) to dye and cytochrome, and substracting gives:

$$Em\ (cyt) = Em\ (dye) + 59 \log (Keq)$$

Electron transport chains

Electron tansport chains are the biological systems to obtain energy by oxidizing substrate. For example, in eukaryotes glucose is oxidized to water and CO_2. On the other hand, bacterias are very versatile and can use a large number of electron donors and acceptors instead of glucose and oxygen. Alternative electron acceptors include nitrate (NO_3^-), nitrite (NO_2^-), fumarate ($C_4H_2O_4^{2-}$) and even H^+.

Energy is conserved by electron transport chains, which accept the electron from the donor, and transfer them sequentially through a series of redox proteins of successively higher redox potential. While conserving some of the energy released by these steps. To be "kinetically competent" in the elctron transport chain, a redox protein should ideally have a midpoint potential between that of its reductant and oxidant so that it can easily be reduced by the reductant, and can easily reduce the oxidant. For example, mitochondria cytochrome c which functions between bc1 complex and cytochrome oxidase has a midpoint potential of 257 mV. While cytochrome c2 of photosynthetic bacteria (which functions between another bc1 complex and photosynthetic reaction center) has a midpoint potential of 360 mV.

Absorbtion spectra of redox proteins

Redox proteins tend to have redox cofactors with delocalized orbitals in order to easily transfer electrons. These delocalized orbitals usually result in absorbtion in the UV or visible range, and the spectrum changes with the redox state (oxidized vs. reduced) of the cofactor. Therefore, UV/visible spectroscopy is a powerful tool to monitor the redox state of the cofactor.

Extinction Coefficient

This is the Beer's Law proportionality constant between concentration and absorbance, as shown below:

$$A_\lambda = C*E_\lambda$$

Where A_λ is the absorbance, C in the concentration of the measured solution, and E_λ is the extinction coefficient. It applies for each compound, at each wavelength in the spectrum. The extinction coefficient at each wavelength is characteristic for the specific compound. For example, the extinction coefficient at a given wavelength is (in general) different for the oxidized and reduced form of the cofactor. A plot of absorbance versus wavelength is called a spectrum. If the compound is in unit concentration, the absorbance at any wavelength is equal to the extinction coefficient at that wavelength. If we have a spectrum of the compound at a known concentration, we can derive the extinction coefficient at any wavelength from Beer's Law ($E_\lambda = A_\lambda/C$.) If we know the extinction coefficient at any one wavelengths, we can calculate the concentration of the compound, and derive the extinction coefficient at any other wavelength as above.

In the experiments described here, the concentration of the cytochrome c solution was determined by pyridine hemochrome analysis (see below). A spectrum of the native cytochrome was taken and divided by concentration to put the spectrum on an extinction coefficient scale, ie the value of the spectrum at any wavelength.

Analyzing a mixture of absorbing compounds

Beer's Law can also be used to analyze a mixture of absorbing compounds by applying it at multiple wavelengths. If we know the spectra of the pure compounds, and we take a spectra of the mixture, we can use simultaneous equation to calculate the concentration of each compound in the mixture. As shown in the equation below (Ci is 1^{st} to last compound):

$$A_\lambda = \Sigma\ Ci*E_\lambda$$

This problem can be solved using the method of simultaneous equations (generalized matrix inversion) to obtain the concentration of each species. In the case where one chemical species is interconverted between two forms (oxidized/reduced), it is sometimes convenient to analyze the spectra in terms of one absolute spectrum (fully reduced), and one difference spectra (oxidized –reduced). These two spectra "span the same space" as the oxidized and reduced spectra, so can fit all the same experimental spectra.

In the experiment described here, we used this method to analyze a mixture of cytochrome c552 and redox indicator dyes to determine the concentration of oxidized/reduced form of each. In the case of one indictator dye (TMPD), the experimental spectra were fit using a spectrum of untreated (mostly reduced) TMPD and a difference spectrum (oxidized-reduced) of the dye.

Cytochrome

A cytochrome is an electron transfer protein whose redox cofactor is a heme. Heme is the cofactor that makes hemoglobin red, although in that case it is functioning to bind oxygen, instead of transferring electron. The reduce form of a low-spin cytochrome has three characteristic absorption bands, calle the alpha, beta, and gamma bands. The gamma band is also called soret band. The wavelengths of the alpha band is characteristic for the type of

low-spin cytochrome (a,b,c). C type cytochrome have a alpha band around 550 nm. The oxidized form of cytochrome has broad nondistinct absorbtion bands.

Pyridine Hemochrome Analysis

The spectrum of a cytochrome depends on the environment of the heme in the protein in a complicated way we cannot predict. In order to determine the concentration of a new cytochrome we convert the heme into a known compound, and then determine its concentration. When a cytochrome is dissolved at high pH, the protein unfolds so the heme is exposed. If there is a high concentration of pyridine in the solution, a complex known as pyridine hemochrome is formed. The spectrum of this complex is characteristic of the type of heme (heme a,b,c,o) and the extinction coefficient is known at least for type c. Thus we can calculate the concentration by Beer's Law. Assuming the conversion to hemochrome is quantative, there is one heme per cytochrome, then this gives us the concentration of the cytochrome in the cuvet, from which we can calculate the concentration of cytochrome in our stock solution.

For c type cytochrome, the heme is ring is covalently bond to the protein. However, we assume the protein does not affect the spectrum.

Protein structure and x-ray crystallography

Proteins consist of polypeptides which are polymers of amino acids connect by amide bonds between amino groups and carobxylate groups. The polypeptide fold in characteristic ways stabilized by hydrogen bonds among other intramolecular forces. In particular, Secondary structure refers to local folding patterns with characteristic hydrogen bonding.

The most practical way in determining protein structure in general, is by x-ray crystallography. This requires crystallizing the protein, which is causing the molecule to aggregate on a precise lattice so that the protein structure is repeated over and over again in the crystal (unit cell). This lattice of protein atoms diffract an x-ray beam into many little beamlets coming off at different angles from the crystal. The relative intensity of the different beamlets is determined by the distirbution of electrons within the unit cell of the crystal.

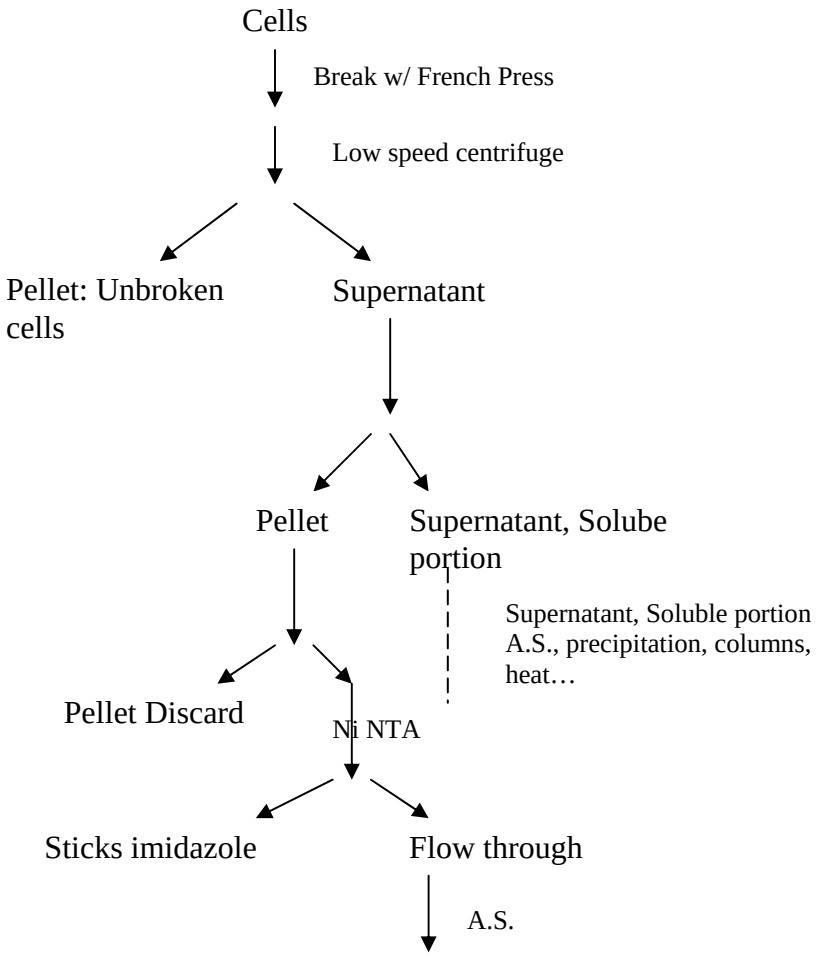
Methods and Materials

Cell growth

Toxin deficient strain of vibrio cholerae (obtained from Robert Gennis, University of Illionois, Urbana-Champaigne) was grown and harvested. The growth media used was LB media, which was made from mixing tryptone (10 g/L), yeast extract (5 g/ L) and NaCl (10 g/L). Antibiotics Ampicillin (100 mg/ml) and Streptomycin (100 mg/ml) were each added in 1ml/L. Arabinose was added to induce gene expression of c552. Next, *Vibrio Cholerae* was innoculated into 5 ml LB and grown overnight at 37 °C, shaken at 200 rpm. It was then plated on agar plate and again grown overnight at 37 °C, shaken at 200 rpm. Cells were harvested by centrifugation.

Protein purification

To harvest the *vibrio cholerae*, protein purification was done by following lab protocol. Since protein purification takes continuous monitoring throughout the day and for several days, it was done in collaboration with Li-Shar Hwang of the Berry group. The general flow chart is as follow:



Standard spectrum of c552 and mitochondrial cyt c

Standard spectrum of pure native c552 and mitochondrial cyt c (hourse heart) were obtained. This includes characteristic of the cytochrome, including its extinction coefficient and its reduced/oxidized form. Dilution of each cytochrome were made and its spectra taken using Shimadzue spectroscopy (paramters of 300-650 nm at low speed to avoid distortion). C552 was added to 1.5 ml 50 mM Kpi until some color was seen. 1 ml was put in cuvet to take spectrum, and the soret peak height (A~413) was reached. 0.1 mM cyt c solution was obtained by weighing out its solid (obtained from sigma/aldrich research) of 0.0058 g in 4.7 ml 50 mM Kpi. An oxidizing agent, FiCN, was added to obtain cytochrome's oxidized form. Dithionite was added after it to obtain the reduced form. A spectrum of the Holmium Oxide filter was taken to verify wavelength calibration.

Extinction Coefficient of c552

In order to put the spectra of c552 on an extinction coefficient scale, pyridine hemochrome spectrum were taken to measure concentration of the c552 working solution. Spectral parameter was 480-630 nm. 0.5 ml of c552 was added to 0.5 ml of pyridine buffer (400 ml/L pyridine, 0.2 M NaOH). In this buffer c552 unfolds and the heme is converted to pyridine hemochrome. Dithionite was subsequently added to ensure pyridine hemochrome in its reduced

form and a series of spectra was scanned. No significant decay of the hemochrome was observed in the first 3 spectra. So the 1st spectra was used to calculate the concentration of c552. This spectrum was fit using a standard spectrum of 10 uM pyridine hemochrome c. The concentration in the cuvet was obtained from the amount of standard spectrum used to fit the experimental spectrum.

Standard spectra of TMPD, DCIP in oxidized and reduced form. As described in background, if spectra of the pure compound is known, the composition of a mixture can be obtained from spectra. In order to determine oxidized and reduced dye and cytochrome, we obtained spectra of TMPD and DCIP in the oxidized and reduced form (spectra of c552 was obtained as described above).

TMPD. Spectrum of the fully reduced form, was obtained after diluting TMPD in 50 mM Kpi buffer and adding a small amount of DT. TMPD is available in the reduced form. Its solution is slowly auto-oxidizable by oxygen. The fully oxidized form could not be obtained, because excess oxidant causes TMPD break down. The difference spectrum, oxidized-reduced, was obtained by diluting TMPD to 50 μM in 50 mM Kpi buffer and taking spectra before and after successive additions of Potassium Ferricyanide. After excess FiCN was added, the absorption began to decrease, indicating TMPD was fully oxidized and began to break down. The last spectra before this was taken (as the almost fully oxidized) spectrum and the first spectrum (almost fully reduced) was subtracted to give the oxidized-reduced difference spectra.

DCIP. Used 5 ul 10 mM DCIP diluted in 1 ml 50 mM Kpi, final concentration of 50 uM. Spectrum was taken at a range of 300-650 nm, and used as the standard spectrum.

Equilibration of c552 with TMPD.

Equilibration constant of c552 with TMPD was determined at 3 different pHs, 6.5 (20 mM MES/100 mM NaCl), 7.5 (20 mM HEPES/100 mM NaCl), and 8.5 (20 mM Tris/100 mM NaCl). The spectrum range was 340-700 nm. 10 ul of c552 was diluted into each of the buffer. The redox indicator dyes TMPD (uM) and DCIP (uM) were added. A spectrum was taken then the redox potential was adjusted negatively using hydroquinone, and ascrobic acid. At low pH, hydroquinone was not a effective reductant, therefore an excess of TMPD was added instead. And then in the positive direction by using FiCN or the oxygen already dissolved in the buffer (TMPD is slowly auto-oxidizable). Finally dithionite was added to get the most reduced state.

The experimental spectra were analyzed to determine the amount of oxidized/reduced cytochrome and redox dye as described in the background.

In the case of one indicator dye (TMPD), the experimental spectra were fit using a spectrum of untreated (mostly reduced) TMPD and a difference spectrum (oxidized-reduced) of the dye. The amount of difference spectrum (Δ) used in the fit ranged from a negative number (Δ_{min}) for fully reduced TMPD to a positive number (Δ_{max}) for fully oxidized. These values were determined or estimated and the ratio of oxidized/reduced form was calculated using the equation

$$[Dye_{ox}]/[Dye_{red}] = (\Delta - \Delta_{min}) / (\Delta_{max} - \Delta)$$

Results

I. **A.** Spectra of cytochrome c552 in the oxidized and reduced form. Figure 1 shows standard spectra of c552. In the oxidized form (red), there is a large soret peak at 415 nm. In the 500-600 nm range, there is a broad double peak called the alpha and beta peaks. In the reduced form (yellow), the soret peak shifts to 417.4 nm, while the alpha and beta peak sharpen up. The alpha peak becomes 550.3 nm and the beta peak becomes 521.8 nm with shoulders on both sides. The difference spectrum (reduced-oxidized) is shown in green. It is important to note that although the protein is called c552 based on the spectrum of its homolog in *Pseudomonas Nautica*, the vibrio species lambda max is actually 550.1 nm.

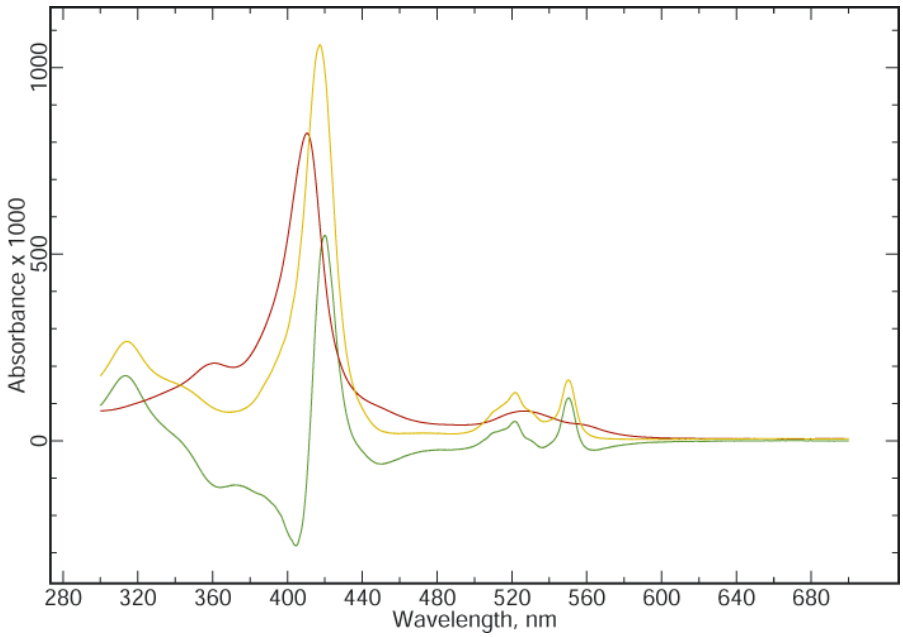


Figure 1. cyt c552 oxidized form (red), reduced form (yellow) and difference spectrum (green).

B. Spectra of mitochondrial cytochrome c for comparison. Figure 2 shows spectra of horse heart cyt c. The oxidized form (yellow), and reduced form (violet) are superficially similar to those of c552. The reduced form of c552 is plotted for comparison (red). Beta peak is relatively higher than the alpha peak in c552.

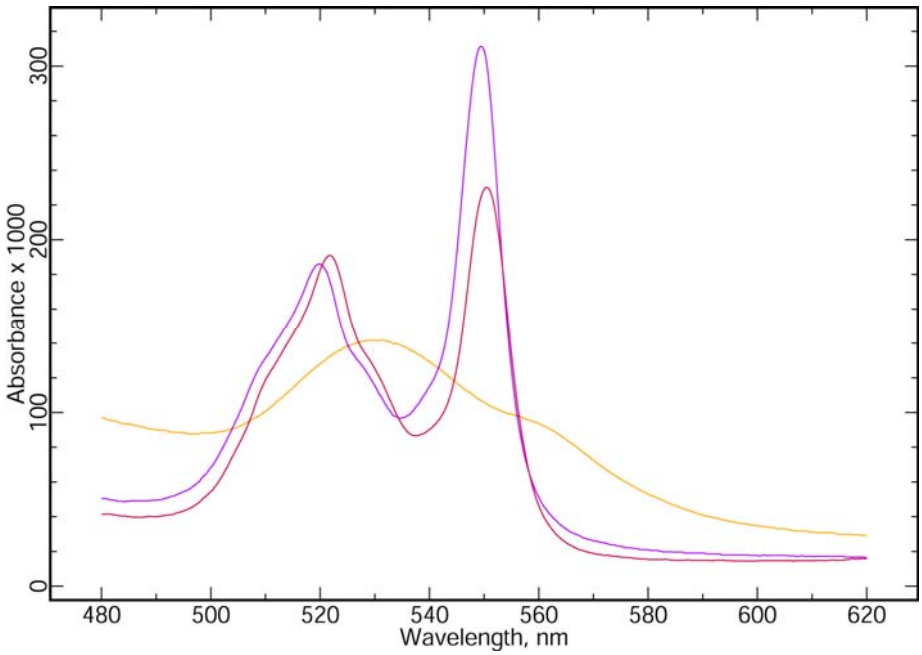


Figure 2.hh cc 10 μM oxidized form (yellow), reduced form (violet), and c552 reduced form (red) is plotted for comparison.

C. Standard spectra of DCIP. DCIP was diluted in 50 mM Kpi buffer and the spectra of the untreated (oxidized) form was taken (blue trace). Then dithionite was added to obtain the fully reduced form of DCIP (green trace). The off scale of the green trace below 360 nm is due to dithionite absorbance.

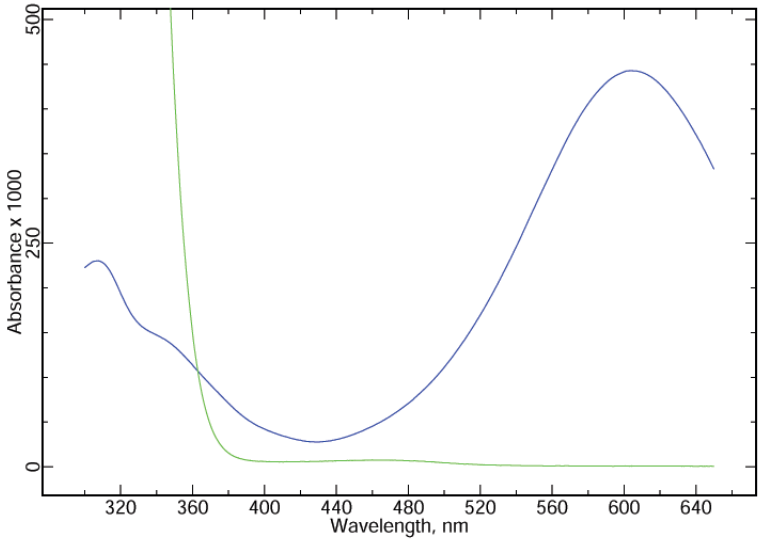


Figure 3. DCIP before (blue) and after (green) adding dithionite.

D.Standard spectra of TMPD. TMPD was diluted into 50 mM Kpi buffer and taken a spectrum (green trace) where it was mostly reduced. In the subsequent traces (cyan, red and violet) TMPD was oxidized by oxygen in the buffer. (Hydroquinone was added before the second trace to see if it will reduce TMPD, which it did not.) At last, the addition of DT fully reduced TMPD, resulting in the disappearance of the two sharp peaks (orange trace).

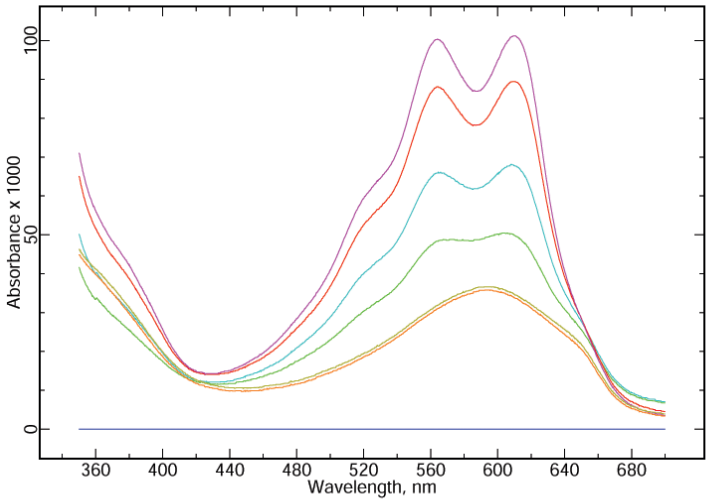


Figure 4. spectra of TMPD in the partially oxidized state (green), subsequently more oxidized (cyan, red and violet) and fully reduced (orange).

II. **A.** *Equilibration of c552 with TMPD at pH 6.5*. Figure 5 shows spectra taken during the redox titration at different redox state. The initial spectrum is the one with no sharp peak at 550 nm, which reperesents the oxidized form of c552. Hydroquinone had no effect presumably because it is a weaker reductant at low pH. Ascorbic Acid reduced DCIP but did not significantly reduce c552. Later TMPD was added (green trace). TMPD is mostly in its reduced form, which has a single broad, weak peak 590 nm. However, there is already some oxidized TMPD, resulting in small peaks at 560 nm and 610 nm . Also cytochrome c552 is partly reduced by TMPD, resulting in a peak at 550 nm. Subsequently, as TMPD goes oxidized by oxgyen dissolved in the buffer (traces from green trace up to light blue trace), the two peaks due to the oxidized state of TMPd gets bigger and bigger, while the 550 nm peak due to the reduced form of c552 gets smaller. Finally, when dithionite is added (brown trace), the two peaks of the oxidized state of TMPD are completely gone, and the c552 alpha and beta peaks are at its largest.

These spectra were analyzed to determine the concentration of each species and the results are shown in Table 1. Each row corresponds to one of the spectra taken. The ratio of oxidized/reduced c552 and TMPD are shown in the appropriately labeled columns. And the equilibrium constant is calculated from each spectrum, is at the right most column. The most reliable values are from those spectra where both cytochrome and dye are close to their midpoint potentials, ie not all the way reduced or oxidized. In this case, traces 11-14 looked the best, they all gave an equilibrium constant around 30. From the Nernst equation, this means the midpoint potential of c552 is 59*log(30) or 87 mV more negative than that of TMPD. The midpoint potential of TMPD is 276 mV, giving 189 mV for c552.

The log of the ratio oxidized/reduced c552 (y) is plotted against the ratio oxidized/reduced of TMPD (x) in Figure X. A slope of 1 on this log plot, implies a 10 fold change in the ratio of c552 accompanies a 10 fold change in the ratio of TMPD, as expected if the equilibrium constant holds. The slope was slightly less than 1 over most of the range, due to some unidentified systematic error. The one outlier is trace 25 after

adding some dithionite. In the spectrum TMPD is more than 95% reduced so its oxidized/reduced ratio is not very accurate, being highly sensitive to error in the footage fully reduced level assumed. However, the traces 11-14 should be reliable.

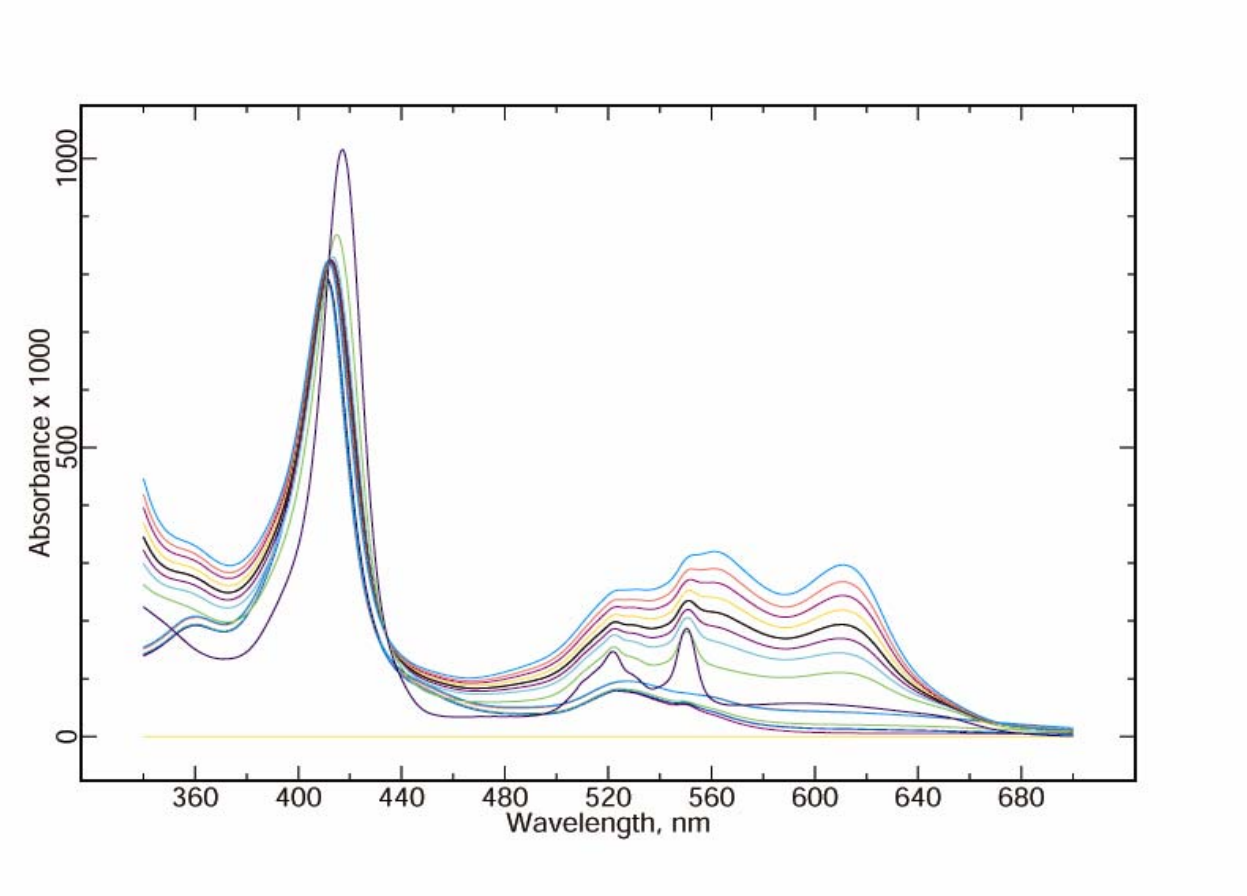


Figure 5. Equilibration of c552 with TMPD at pH 6.5. 10 ul c552 was diluted in 20 mM MES 6.5 buffer. After the first scan was taken (violet?), then 10 mM DCIP, 10 mM hydroquinone and 10 mM Ascrobic acid were added sequentially while taking spectra. Then TMPD was added (green trace), and spectra were recorded at intervals while TMPD went oxidized. After the last of these spectra (light blue), dithionite was added resulting in the brown trace.

Table 1-equilibration of c552 with TMPD at pH 6.5

Spectrum taken	$c552_O$	$C552_R$	$DCIP_o$	$TMPD_{mr}$	$tmpd_{diff}$	RMS error	c552 o/r	Log $c_{O/R}$	TMPD $_{O/R}$	Log $T_{O/R}$	Keq
1. 20 mM MES 6.5	0.00	0.00	0.00	0.00	0.00	0.00					
2. +10 ul cyt 552	0.89	0.08	0.00	0.00	0.00	0.04	11.64	1.07			
3. + 0.5 ul DCIP	0.97	0.02	0.07	0.23	-0.01	0.12	53.94	1.73			
4. + 2 ul BQH2	0.96	0.02	0.07	0.12	0.00	0.09	41.57	1.62			
5. + 1 uL 10 mM Asc acid	0.92	0.05	0.05	-0.13	0.00	0.05	17.26	1.24			
6. repeat	0.91	0.07	0.02	0.04	0.00	0.06	13.71	1.14			
7. repeat	0.91	0.07	0.02	0.05	0.00	0.06	13.71	1.14			
8. + 5 ul TMPD	0.60	0.36	-0.01	2.02	0.11	0.10	1.66	0.22	0.05	-1.33	35.31
9. repeat	0.66	0.30	0.00	2.01	0.17	0.08	2.18	0.34	0.07	-1.17	32.57
10. repeat	0.70	0.26	0.00	2.01	0.22	0.08	2.71	0.43	0.09	-1.06	31.25
11. repeat	0.74	0.22	0.00	2.03	0.27	0.09	3.30	0.52	0.11	-0.97	30.73
12. repeat	0.77	0.20	0.00	1.92	0.33	0.09	3.93	0.59	0.13	-0.88	29.83
13. repeat	0.79	0.17	0.00	1.93	0.38	0.10	4.51	0.65	0.15	-0.81	29.35
14. repeat	0.81	0.15	0.00	1.90	0.44	0.10	5.33	0.73	0.18	-0.74	29.42
15. + DT (small amnt)	0.44	0.46	0.04	1.02	0.08	0.29	0.95	-0.02	0.04	-1.45	27.08
16. + DT (Excess)	0.06	0.90	0.02	1.29	-0.03	0.21	0.06	-1.21	0.00		

$TMPD_{mr}$ TMPD,mostly reduced

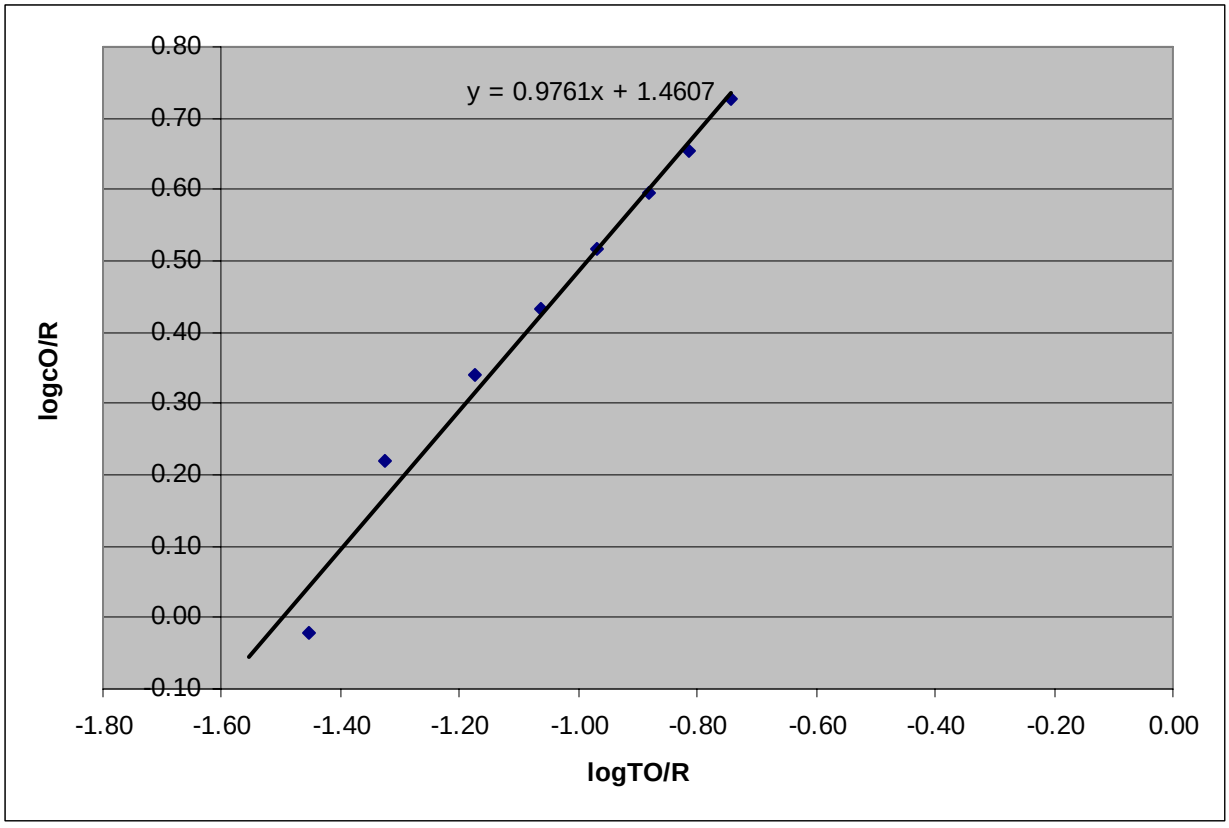


Figure 6. redox equilibration of c552 with TMPD. Log of oxidized/reduced c552 ratio (y), log of oxidized/reduced TMPD ratio (x). If equilibrium holds, a change in the oxidized/reduced ratio of c552 by a certain factor will be accompanied by a change of the same factor in the ratio of TMPD. Therefore, the log-log plot should have a slope of 1, and intersect the x axis at the -log of equilibrium constant.

B. *Equilibration of c552 with TMPD at pH 7.5 1st trial*. (10/25) In all the experiments, we started with c552 in the oxidized form. It is necessary to get c552 partly reduced to get the equilibrium constant. In addition of TMPD, giving some reductant of c552. In this trial, we tried to use hydroquinone to adjust the redox potential, but it turned out hydroquinone did not significantly reduce c552, meaning that the midpoint potential of c552 is more negative than we expected. However, TMPD did reduce a significant amount of c552, so we were able to calculate the equilibrium constant. In later experiments higher concentration of TMPD was used to obtain better reduction of c552.

The equilibrium constant for this experiment was around 10 obtained from trial 4-8. Hydroquinone did not further reduce TMPD, but after adding dithionite TMPD was fully reduced so the oxidized/reduced ratio could not be calculated. Also adding ferricyanide subsequently reoxidized the c552 but did not reoxidize TMPD, prehaps because ferricyanide over-oxidizes TMPD to its unstable doubly oxidized form.

An equilibration constant of 10, this means the midpoint potential of c552 is 59*log(10) or 59 mV more negative than that of TMPD. The midpoint potential of TMPD is 276 mV, giving 217 mV for c552.

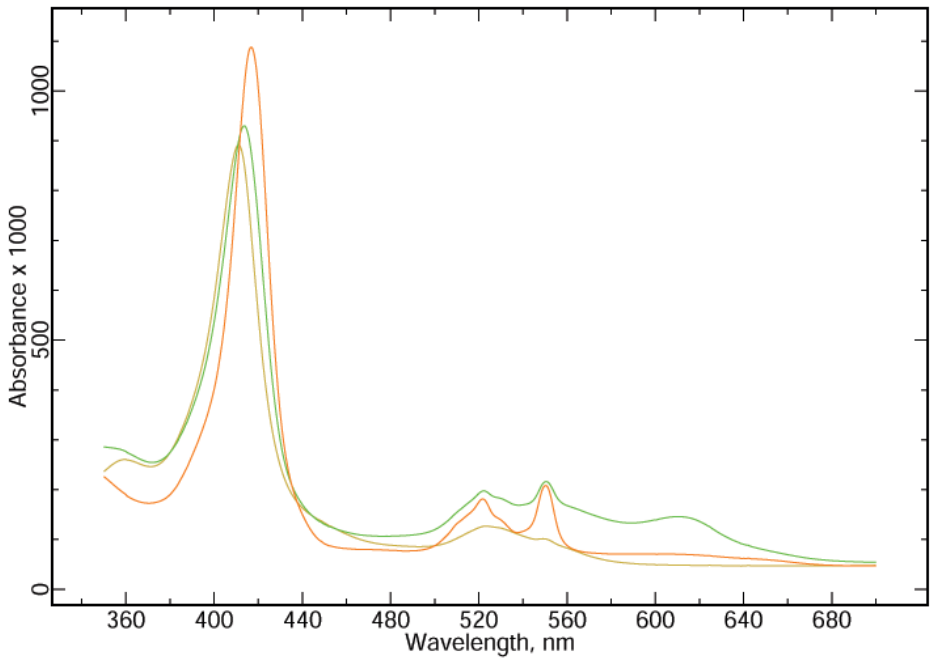


Figure 7. Equilibration of c552 with TMPD at pH 7.5 1st trial. At first c552 was diluted in 50 mM Kpi buffer (brown trace), then DCIP, TMPD were added (green trace). Hydroquinone was added but it had no effect (not shown). Finally, dithionite was added (orange trace).

Table 2-equilibration of c552 with TMPD at pH 7.5 1st trial

Spectrum taken	$c552_O$	$C552_R$	$DCIP_o$	$TMPD_{mr}$	$tmpd_{diff}$	RMS error	c552 o/r	Log $c_{O/R}$	TMPD $_{O/R}$	Log $T_{O/R}$	Keq
1. 20 mM HEPES pH 7.5	0	0	0	0	0	0					
2. +10 ul cyt 552	0.94	0.08	0.00	0	-0.00	0.04	11.43	1.06			
3. + 0.5 ul DCIP	0.92	0.010	0.12	-0.20	0.01	0.05	9.60	0.98			
4. + 1.5 ul TMPD	0.62	0.39	0.03	0.62	0.12	0.11	1.58	0.20			
5. repeat	0.63	0.38	0.03	0.61	0.12	0.1	1.63	0.21	0.16	-0.80	10.19
6. repeat	0.64	0.37	0.03	0.60	0.13	0.09	1.74	0.24	0.17	-0.76	10.14
7. 2 ul hydroquinone	0.63	0.37	0.036	0.45	0.13	0.104	1.71	0.23	0.18	-0.76	9.74
8. repeat	0.67	0.33	0.04	0.56	0.14	0.08	1.99	0.30	0.20	-0.71	10.13
+ 10 ul DT	0.15	0.86	0.00	0.46	0	0.15	0.17	-0.76			
repeat	0.11	0.91	-0.00	0.53	-0.01	0.16	0.12	-0.93			
Mix w/ pipette	0.11	0.92	0.03	-0.01	0.01	0.19	0.12	-0.93			
repeat	0.10	0.92	0.00	0.48	-0.01	0.17	0.11	-0.97			
+ 1ul FiCN	0.27	0.74	0.02	0.26	0.02	0.18	0.36	-0.44			
repeat	0.15	0.86	0.00	0.50	0.00	0.16	0.17	-0.76			
repeat	0.29	0.71	0.02	0.14	0.02	0.18	0.41	-0.39	0.03		
repeat	0.16	0.86	0.00	0.44	0.00	0.15					
+ 1ul FiCN	0.39	0.60	0.03	0.08	0.04	0.26					
repeat	0.21	0.80	-0.01	0.50	0.00	0.15					
+ 1ul FiCN	0.43	0.55	0.04	-0.17	0.05	0.29					
repeat	0.22	0.78	-0.01	0.52	0.00	0.16					
+ 1ul FiCN	0.47	0.50	0.02	0.02	0.05	0.35					
repeat	0.28	0.73	-0.01	0.54	0.00	0.16					
2 ul FiCN (??)	0.59	0.39	0.02	0.05	0.02	0.26					
mix and repeat	0.40	0.60	-0.02	0.57	0.00	0.18					
+ 2 uL FiCN	0.85	0.13	0.02	0.13	0.00	0.16					
repeat	0.75	0.24	-0.01	0.49	-0.01	0.17					
Repeat later	0.67	0.32	-0.02	0.58	-0.01	0.18					

$TMPD_{mr}$ TMPD,mostly reduced

C. *Equilibration of c552 with TMPD at pH 7.5 2nd trial*. For this experiment, the procedure was as in the previous experiment (B.), but a larger quantity of TMPD was used (300 uM). This resulted in a greater level of c552 reduction. After TMPD was added, it was gradually oxidized by oxygen in the buffer. Ascrobic acid was also added (trace 9 in Table 3.), and temporarily reversed the oxidation but was unable to reach a high level of reduction. After no more ascorbic acid was added (trace 16 in Table 3.), the mixture went further oxidized. Finally, dithionite was added, and it completely reduced c552 and TMPD.

The equilibrium constant for this experiment was ranged from 18 to 25. The value varied systematically with redox level giving higher values under the more oxidized conditions. Between spectra 9-14 the redox level was maintained approximately the same, and the value obtained in this range was 20. Afterwards when the redox level went more oxidized the value increased to 25 before we added dithionite. Possible reason for variation is due to uncertainty of

reduce end point of TMPD, which causes less error when TMPD is more oxidized. If this is the case, the values obtained under the more oxidizing conditions are more accurate.

With an equilibration constant of 20, his means the midpoint potential of c552 is 59*log(20) or 77 mV more negative than that of TMPD. The midpoint potential of TMPD is 276 mV, giving 199 mV for c552.

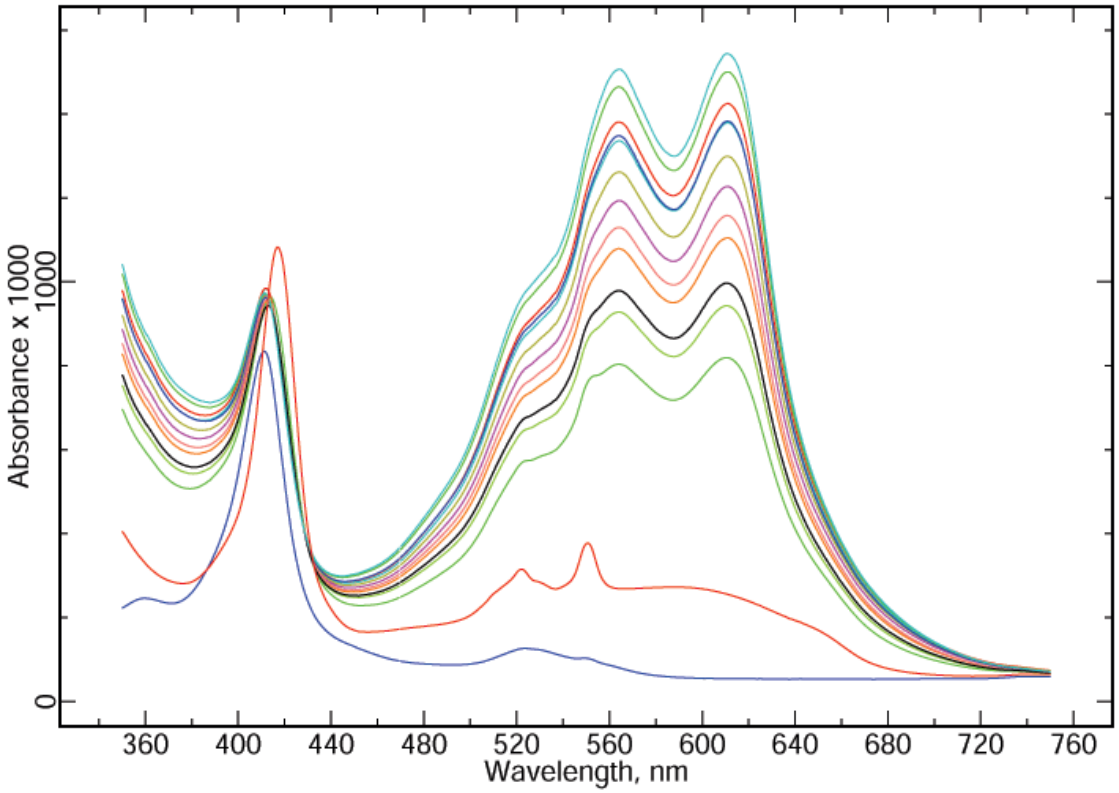


Figure 8. equilibration of c552 with TMPD at pH 7.5 2nd trial. C552 was diluted in 50 mM Kpi (trace blue). TMPD and DCIP were added (lower green trace). The redox level oscillated but gradually went oxidized until the upper cyan trace during four additions of ascorbic acid. Then dithionite was added (red trace).

Table 3-equilibration of c552 with TMPD at pH 7.5 2nd trial

	$c552_O$	$C552_R$	$DCIP_o$	$TMPD_{mr}$	$tmpd_{diff}$	RMS error	c552 o/r	Log $c_{O/R}$	TMPD $_{O/R}$	Log $T_{O/R}$	Keq
1. 20 mM HEPES pH 7.5	0.00	0.00	0.00	0.00	0.00	0.00					
2. +10 ul cyt 552	0.88	0.07	0.01	-0.05	0.00	0.08	11.92	1.08			
3. + 0.5 ul DCIP	0.91	0.05	0.87	0.36	-0.01	0.13	18.94	1.28			
4. + 15 ul TMPD	0.61	0.34	0.29	5.84	0.80	0.55	1.79	0.25	0.11	-0.95	16.11
5. repeat	0.61	0.33	0.33	5.81	0.88	0.55	1.87	0.27	0.12	-0.92	15.45
6. repeat	0.63	0.31	0.36	5.91	0.94	0.56	2.01	0.30	0.13	-0.89	15.48
7. repeat	0.67	0.29	0.41	5.96	1.05	0.58	2.30	0.36	0.15	-0.84	15.78
8. repeat	0.74	0.25	0.49	5.81	1.21	0.57	2.93	0.47	0.17	-0.77	17.33
9. + 1 ul 10 mM Asc Acid	0.88	0.20	0.67	5.91	1.65	0.77	4.53	0.66	0.24	-0.62	18.97
10. repeat	0.92	0.18	0.69	5.88	1.73	0.79	5.12	0.71	0.25	-0.60	20.28
11. + 10 ul 10 mM Asc Acid	0.84	0.22	0.55	6.11	1.41	0.67	3.85	0.59	0.20	-0.70	19.31
12. repeat	0.86	0.21	0.59	6.22	1.52	0.74	4.15	0.62	0.22	-0.66	19.15
13. + 10 ul Asc Acid	0.80	0.24	0.45	6.26	1.22	0.57	3.30	0.52	0.17	-0.77	19.33
14. repeat	0.83	0.23	0.49	6.27	1.30	0.60	3.67	0.56	0.18	-0.74	20.02
15. + 10 ul Asc Acid	0.71	0.29	0.31	6.65	0.97	0.54	2.43	0.38	0.13	-0.87	18.08
16. repeat	0.75	0.28	0.35	6.60	1.05	0.56	2.73	0.44	0.15	-0.84	18.70
17. repeat	0.95	0.18	0.59	6.53	1.67	0.84	5.27	0.72	0.24	-0.62	21.78
18. repeat	1.03	0.15	0.64	6.63	1.87	0.94	6.99	0.84	0.28	-0.56	25.20
19. repeat	1.05	0.14	0.65	6.73	1.94	1.01	7.45	0.87	0.29	-0.54	25.67
20. + DT	0.17	0.82	-0.15	6.68	-0.11	0.48	0.21	-0.68	0.00	#NUM	#DIV/0
21. repeat	0.22	0.79	-0.17	6.98	-0.09	0.51	0.28	-0.55	0.00	-2.84	197.58

$TMPD_{mr}$:TMPD,mostly reduced
Asc Acid: Ascrobic Acid

D. *Equilibration of c552 with TMPD at pH 8.5*. After adding TMPD and DCIP, c552 was partially reduced, allowing the equilibrium constant to be calculated. Immediately after the hydroquinone additions, a slightly higher value was obtained (15, 18) due to a greater reduction of TMPD than c552. This may be a kinetic affect and c552 is not in equilibrium with TMPD, so the values around 12 are more reliable. Otherwise the value of the equilibrium constant range from 10 to 12, while the ratio of oxidized/reduced c552 went from 1 to 15, and oxidized/reduced TMPD went from 0.05 to 1.4. This constant value over a wide range of redox levels increases the value's credibility.

With an equilibration constant of 12, his means the midpoint potential of c552 is 59*log(12) or 64 mV more negative than that of TMPD. The midpoint potential of TMPD is 276 mV, giving 212 mV for c552.

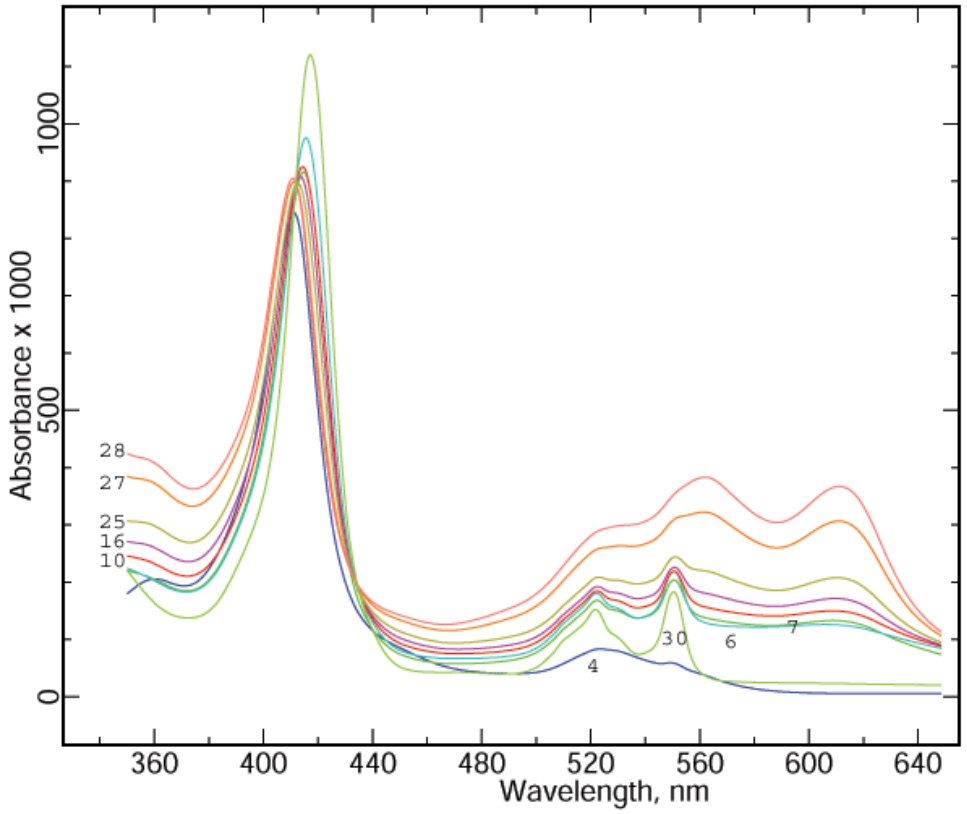


Figure 9. 5 ul c552 was added to 1 ml of 50 mM Kpi buffer (trace 4), DCIP and TMPD were added (trace 6), in traces 6~28, TMPD was gradually oxidized by oxygen with temporary reduction by hydroquinone addition in traces 7 and 11. Finally, dithionite was added in trace 30.

Table 4-equilibration of c552 with TMPD at pH 8.5

	$c552_O$	$C552_R$	$DCIP_o$	$TMPD_{mr}$	$tmpd_{diff}$	RMS error	c552 o/r	Log $c_{O/R}$	TMPD $_{O/R}$	Log $T_{O/R}$	Keq
1. 20 mM Tris pH 8.5	0.00	0.00	0.00	0.00	0.00	0.00	#DIV/0!	#DIV/0!			
2. +5 ul cyt 552	0.43	0.04	0.00	0.00	0.00	0.04	10.83	1.03			
3. repeat	0.44	0.04	0.00	0.00	0.00	0.04	10.88	1.04			
4. + 0.5 ul cytc 552	0.95	0.09	0.00	-0.03	0.00	0.07	10.99	1.04			
5. + 0.5 ul DCIP	0.94	0.09	0.15	0.01	0.00	0.08	10.40	1.02			
6. + 1.5 ul TMPD	0.51	0.52	0.15	0.78	0.07	0.12	0.98	-0.01	0.09	-1.07	11.58
7. + 0.5 ul Hydroquinone	0.38	0.67	0.13	0.90	0.02	0.18	0.56	-0.25	0.03	-1.50	17.50
8. repeat	0.45	0.59	0.13	0.89	0.04	0.15	0.76	-0.12	0.05	-1.27	14.25
9. repeat	0.51	0.54	0.14	0.90	0.06	0.13	0.95	-0.02	0.07	-1.13	12.93
10. repeat	0.55	0.50	0.14	0.90	0.07	0.12	1.10	0.04	0.09	-1.04	12.20
11. + 1ul hydroquinone	0.43	0.61	0.13	0.92	0.03	0.17	0.70	-0.15	0.04	-1.35	15.60
12. repeat	0.53	0.51	0.13	0.93	0.06	0.14	1.05	0.02	0.08	-1.08	12.53
13. repeat	0.57	0.47	0.13	0.94	0.08	0.13	1.20	0.08	0.10	-1.00	11.91
14. repeat	0.59	0.44	0.13	0.95	0.09	0.12	1.35	0.13	0.12	-0.93	11.50
15. repeat	0.62	0.42	0.13	0.95	0.10	0.11	1.48	0.17	0.13	-0.87	11.07
16. repeat	0.64	0.39	0.13	0.96	0.11	0.11	1.64	0.22	0.15	-0.82	10.88
17. Repeat	0.66	0.37	0.13	0.97	0.12	0.11	1.76	0.25	0.16	-0.78	10.71
18. repeat	0.67	0.36	0.13	0.97	0.13	0.10	1.87	0.27	0.18	-0.75	10.43
Repeat	0.69	0.35	0.13	0.98	0.14	0.10	1.97	0.30	0.19	-0.72	10.36
Repeat	0.70	0.34	0.13	0.98	0.15	0.10	2.07	0.32	0.20	-0.70	10.26
r	0.71	0.32	0.13	0.99	0.16	0.10	2.21	0.34	0.22	-0.66	10.13
r	0.72	0.31	0.13	0.99	0.16	0.10	2.28	0.36	0.23	-0.65	10.11
r	0.72	0.30	0.12	1.00	0.17	0.11	2.38	0.38	0.24	-0.63	10.08
r	0.73	0.30	0.12	1.01	0.17	0.10	2.46	0.39	0.24	-0.61	10.05
r later	0.75	0.28	0.12	1.04	0.19	0.11	2.69	0.43	0.27	-0.57	9.93
Later, repeat	0.88	0.13	0.11	1.18	0.38	0.13	7.07	0.85	0.74	-0.13	9.61
r	0.89	0.12	0.11	1.18	0.39	0.14	7.62	0.88	0.78	-0.11	9.77
r later	0.93	0.06	0.11	1.14	0.52	0.14	14.94	1.17	1.40	0.15	10.69
29. +DT	0.05	0.99	-0.02	0.14	0.00	0.33	0.05	-1.28	0.01	-2.18	7.86
30. R DT	0.06	0.99	-0.03	0.41	-0.01	0.29	0.06	-1.23	0.00	-2.96	52.81

$TMPD_{mr}$: TMPD,mostly reduced

Discussion

C552 was purified from *vibio cholerae* and characterized in respect to UV/visible absorption spectra and redox midpoint potential. The spectra shows the alpha band of the reduced form is unusually weak compared to a typical type I c ctychrome, such as mitochondrial cytochrome c. The absorption maximum of the alpha band is 551.3 nm. Thus either the name c552 or c553 are misnomers based on the spectra of homologous proteins.

The equilibrium constant for reduction of TMPD by c552 ranged from 10 to 30 with a tendency to be higher at acid pH, that is c552 reduces TMPD to a slightly greater extent at acid pH. If proton uptake accompanies electron uptake by TMPD, the equilibrium constant would be expected to vary 10-fold per pH unit. The pH dependence was much less than 10-fold per pH unit. If in fact the pH dependence is real, it may reflect partial protonation of the reduced form due to a pKa below pH 6.5. If that is the case, the midpoint potential of TMPD at pH 6.5 may be higher than the literature value of +276 mV at pH 7.

The midpoint potential was calculated at each pH by subtracting 59*log(Keq) from the midpoint potential of TMPD, 276 mV. Due to the uncertainty of this value at pH 6.5, the lower Em obtained at 6.5 may be less reliable, and we used the values at 7.5 and 8.5, 200-217 mv.

This midpoint potential is considerably lower than that of mitochondrial cytochrome c (257 mV). This suggests this cytochrome does not function as a electron donor to a terminal oxidase. In fact, attempts to demonstrate oxidation of c552 by vibrio membranes in the presence of oxygen were unsuccessful (not shown). The actual function is not known. But presumably reduces something with a more negative potential than an oxidase. The related protein fron *P. Nautica* has been shown to function as an electron donor to Nitrite reductase, but suspected to have other functions as well. According to Brown at al[1].:

"The monohemic cytochrome c552 from Pseudomonas nautica (c552-Pn) is thought to be the electron donor to cytochrome cd1, the so-called nitrite reductase (NiR). It shows as high levels of activity and affinity for the P. nautica NiR (NiR-Pn), as the Pseudomonas aeruginosa enzyme (NiR-Pa). Since cytochrome c552is by far the most abundant electron carrier in the periplasm, it is probably involved in numerous other reactions. Its sequence is related to that of the c type cytochromes, but resembles that of the dihemic c4cytochromes even more closely."
1: J Mol Biol. 1999 Jun 18;289(4):1017-28

We expect that the function of c552 in vibrio cholerae is similar. The genome of *vibrio cholerae* contains genes that have have been annotated as homologous to nitrite and nitrate reductase of other organisms.

1. MAD structure of Pseudomonas nautica dimeric cytochrome c552 mimicks the c4 Dihemic cytochrome domain association. Brown K, Nurizzo D, Besson S, Shepard W, Moura J, Moura I, Tegoni M, Cambillau C.