3E5. Visible/UV spectroscopy of *bc₁* complexes (background for Aim 5).

Delocalized electron orbital systems in redox centers tend to have distinctive absorbance in the visible or near UV. Real-time monitoring, sensitivity, and the non-destructive nature of Vis/UV spectroscopy make it an attractive method for static analysis of the ***bc₁*** complex or following kinetics. However Vis/UV spectra tend to be broad and overlapping so it is seldom possible to find a single wavelength that will report the redox state of a single cofactor. Since the early post-war experiments of Britton Chance, kinetics of cytochrome systems have been followed mainly by dual-wavelength spectroscopy and concentration determination of cytochromes in samples have been based on reduced-minus-oxidized extinction coefficients at a single wavelength pair, because this information could be obtained directly from an analog instrument. Since cytochrome spectra (and vis-UV spectra in general) tend to be broad and overlapping, There were several attempts at quantitative analysis of mixtures of cytochromes (Williams 1964; Vanneste 1966; Vanneste 1966) in the 50's an 60's, but these efforts were perhaps doomed to neglect by the difficulty of getting data from an analog spectrophotometer into a form readable by a digital computer, as well as the poor availability of computers in those days. Today spectrophotometers are digital instruments capable of saving spectra in a variety of computer-readable formats, and personal computers capable of this type of analysis are as common now than typewriters were then.

If you look at the difference spectra in Figure 6, it is clear that the problem is very well determined, that is, there is only one way these spectra could be combined to obtain a given observed spectrum. In principle one could manually adjust the concentration of each component to obtain the observed spectrum. Now suppose you put a few percent too much of one cytochrome, there would be a bad fit in the region of the peak of that cytochrome, and no amount of twiddling the other concentrations would fix it. The solution is very robust. One can set up systems of simultaneous equations relating absorbance at n wavelengths to concentrations of n components. Inclusion of 1-3 more wavelengths allows correction for baseline offset, slanted, or gently curved baseline changes. (Berry and Trumpower 1987). However linear least squares analysis using the entire spectrum will give us the set of concentrations that fits best in a single non-iterative step that takes a fraction of a second. The algorithm(Sternberg, Stillo et al. 1960) was described long ago

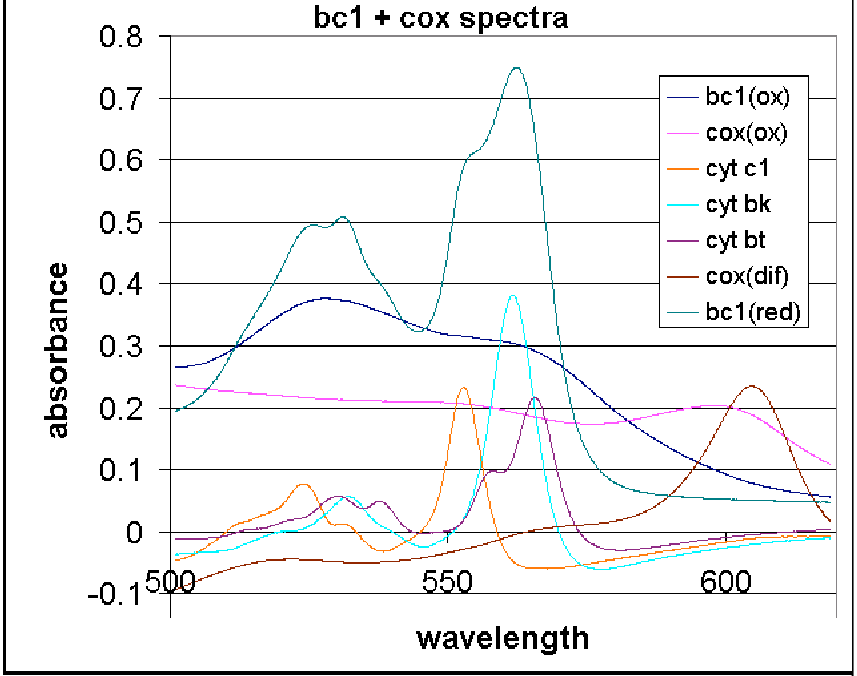

Figure 6. Absolute and difference spectra of cytochromes.

when digitized spectra and computer time were a lot harder to come by. The main difficulty is in obtaining a set of pure difference spectra of each component with which to fit the unknown, and putting those spectra on an absolute extinction coefficient basis.

Assuming the chromophores cannot be physically separated without altering their spectral properties, the best way is to systematically alter the concentrations in a predictable (model-able) way, and obtain extinction coefficients at each wavelength as (linear) parameters from fitting the model. This could involve redox titration as we used in (Berry, Huang et al. 1991), or simple kinetic or equilibrium experiments.

Recently such a deconvolution technique has been applied to ***bc$_1$*** complex of *Rb. sphaeroides* by Shinkarev and coworkers(Shinkarev, Crofts et al. 2006; Shinkarev, Crofts et al. 2006) with encouraging results. Their application is a particularly difficult one as they are fitting microsecond kinetics and the data is obtained by flash spectroscopy one wavelength at a time. This limits the number of wavelengths that can be acquired, and choice of wavelengths becomes important. For more normal applications where entire spectra can be recorded for each datum, this can be avoided and the optimum wavelengths will necessarily be included by using all wavelengths. Since we are working with large amounts of ***bc$_1$*** complex from various organisms, we are in a good position to obtain the standard basis spectra for use in such methods with little or no extra effort or expense, and this is what we propose to do in Aim 5.

References:

Berry, E. A., L. S. Huang, et al. (1991). "Ubiquinol-cytochrome c oxidoreductase of higher plants. Isolation and characterization of the bc1 complex from potato tuber mitochondria." J Biol Chem **266**(14): 9064-77.

A procedure is described for isolation of active ubiquinol-cytochrome c oxidoreductase (bc1 complex) from potato tuber mitochondria using dodecyl maltoside extraction and ion exchange chromatography. The same procedure works well with mitochondria from red beet and sweet potato. The potato complex has at least 10 subunits resolvable by gel electrophoresis in the presence of dodecyl sulfate. The fifth subunit carries covalently bound heme. The two largest ("core") subunits either show heterogeneity or include a third subunit. The purified complex contains about 4 mumol of cytochrome c1, 8 mumol of cytochrome b, and 20 mumol of iron/g of protein. The complex is highly delipidated, with 1-6 mol of phospholipid and about 0.2 mol of ubiquinone/mol of cytochrome c1. Nonetheless it catalyzes electron transfer from a short chain ubiquinol analog to equine cytochrome c with a turnover number of 50-170 mol of cytochrome c reduced per mol of cytochrome c1 per s, as compared with approximately 220 in whole mitochondria. The enzymatic activity is stable for weeks at 4 degrees C in phosphate buffer and for months at -20 degrees C in 50% glycerol. The activity is inhibited by antimycin, myxothiazol, and funiculosin. The complex is more resistant to funiculosin and diuron than the beef heart enzyme. The optical difference spectra of the cytochromes were resolved by analysis of full- spectrum redox titrations. The alpha-band absorption maxima are 552 nm (cytochrome c1), 560 nm (cytochrome b-560), and 557.5 + 565.5 nm (cytochrome b-566, which has a split alpha-band). Extinction coefficients appropriate for the potato cytochromes are estimated. Despite the low lipid and ubiquinone content of the purified complex, the midpoint potentials of the cytochromes (257, 51, and -77 mV for cytochromes c1, b-560, and b-566,

respectively) are not very different from values reported for whole mitochondria. EPR spectroscopy shows the presence of a Rieske-type iron sulfur center, and the absence of centers associated with succinate and NADH dehydrogenases. The complex shows characteristics associated with a Q-cycle mechanism of redox- driven proton translocation, including two pathways for reduction of b cytochromes by quinols and oxidant-induced reduction of b cytochromes in the presence of antimycin.

Berry, E. A. and B. L. Trumpower (1987). "Simultaneous determination of hemes a, b, and c from pyridine hemochrome spectra." Anal Biochem **161**(1): 1-15.

Two procedures for analyzing overlapping optical spectra of mixtures of pyridine hemochromes are described, and extinction coefficients of pyridine hemochromes are provided for use with these methods. In the first procedure, absorbance is measured at a number of wavelengths equal to the number of components to be analyzed. This is the minimum amount of spectral data from which the concentration of each species can be calculated. In the second procedure, absorbance is measured at a number of wavelengths greater than the number of components to be analyzed. This redundancy of information makes it impossible to fit spectra which contain contributions from additional components, unless the spectra of the additional components are equal to linear combinations of the spectra of the species being analyzed. These two procedures are generally applicable to analyses of absolute or difference spectra of mixtures of components obeying Beer's law. The sensitivity to error in the absorbance measurements is only slightly greater than that for measuring a pure component at a single wavelength.

Shinkarev, V. P., A. R. Crofts, et al. (2006). "Spectral analysis of the bc(1) complex components in situ: beyond the traditional difference approach." Biochim Biophys Acta **1757**(1): 67-77.

The cytochrome (cyt) bc(1) complex (ubiquinol: cytochrome c oxidoreductase) is the central enzyme of mitochondrial and bacterial electron-transport chains. It is rich in prosthetic groups, many of which have significant but overlapping absorption bands in the visible spectrum. The kinetics of the cytochrome components of the bc(1) complex are traditionally followed by using the difference of absorbance changes at two or more different wavelengths. This difference-wavelength (DW) approach has been used extensively in the development and testing of the Q-cycle mechanism of the bc(1) complex in Rhodobacter sphaeroides chromatophores. However, the DW approach does not fully compensate for spectral interference from other components, which can significantly distort both amplitudes and kinetics. Mechanistic elaboration of cyt bc(1) turnover requires an approach that overcomes this limitation. Here, we compare the traditional DW approach to a least squares (LS) analysis of electron transport, based on newly determined difference spectra of all individual components of cyclic electron transport in chromatophores. Multiple sets of kinetic traces, measured at different wavelengths in the absence and presence of specific inhibitors, were analyzed by both LS and DW approaches. Comparison of the two methods showed that the DW approach did not adequately correct for the spectral overlap among the components, and was generally unreliable when amplitude changes for a component of interest were small. In particular, it was unable to correct for extraneous contributions to the amplitudes and kinetics of cyt b(L). From LS analysis of the chromophoric components (RC, c(tot), b(H) and

b(L)), we show that while the Q-cycle model remains firmly grounded, quantitative reevaluation of rates, amplitudes, delays, etc., of individual components is necessary. We conclude that further exploration of mechanisms of the bc(1) complex, will require LS deconvolution for reliable measurement of the kinetics of individual components of the complex in situ.

Shinkarev, V. P., A. R. Crofts, et al. (2006). "Spectral and kinetic resolution of the bc(1) complex components in situ: A simple and robust alternative to the traditional difference wavelength approach." Biochim Biophys Acta.

The kinetics of the cytochrome (cyt) components of the bc(1) complex (ubiquinol: cytochrome c oxidoreductase, Complex III) are traditionally followed by using the difference of absorbance changes at two or more different wavelengths. However, this difference-wavelength (DW) approach is of limited accuracy in the separation of absorbance changes of components with overlapping spectral bands. To resolve the kinetics of individual components in Rhodobacter sphaeroides chromatophores, we have tested a simplified version of a least squares (LS) analysis, based on measurement at a minimal number of different wavelengths. The success of the simplified LS analysis depended significantly on the wavelengths used in the set. The "traditional" set of 6 wavelengths (542, 551, 561, 566, 569 and 575 nm), normally used in the DW approach to characterize kinetics of cyt c(tot) (cyt c(1)+cyt c(2)), cyt b(L), cyt b(H), and P870 in chromatophores, could also be used to determine these components via the simplified LS analysis, with improved resolution of the individual components. However, this set is not sufficient when information about cyts c(1) and c(2) is needed. We identified multiple alternative sets of 5 and 6 wavelengths that could be used to determine the kinetics of all 5 components (P870 and cyts c(1), c(2), b(L), and b(H)) simultaneously, with an accuracy comparable to that of the LS analysis based on a full set of wavelengths (1 nm intervals). We conclude that a simplified version of LS deconvolution based on a small number of carefully selected wavelengths provides a robust and significant improvement over the traditional DW approach, since it accounts for spectral interference of the different components, and uses fewer measurements when information about all five individual components is needed. Using the simplified and complete LS analyses, we measured the simultaneous kinetics of all cytochrome components of bc(1) complex in the absence and presence of specific inhibitors and found that they correspond well to those expected from the modified Q-cycle. This is the first study in which the kinetics of all cytochrome and reaction center components of the bc(1) complex functioning in situ have been measured simultaneously, with full deconvolution over an extended time range.

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