Spectroscopic Forms of Carbonmonoxi-Cytochrome Oxidase

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A systematic study of the errors of low-temperature recording of kinetics of the cytochrome oxidase–CO reaction had identified the classic devitrification process of Keilin & Hartree [(1950) *Nature (London)* **165**, 504–505]. The methodology described here minimizes this effect, and the computation methods afford appropriate ways of detecting a residual effect. Thus it has been possible to identify that absorption difference spectra and kinetics of the reaction of fully reduced or half-reduced cytochrome oxidase with CO indicate only one spectroscopic form of the respective carbonmonoxi-cytochrome oxidase.

Cytochrome oxidase (ferrocytochrome *c*-oxygen oxidoreductase, EC 1.9.3.1), the terminal oxidase of the electron-transport chain of all higher organisms, minimally requires two haem groups (*a* and *a*₃) and two copper atoms to function. This enzyme is capable of binding dioxygen (at haem *a*₃) and catalysing its reduction to water.

The reaction of cytochrome oxidase with CO has attracted considerable attention (Sharrock & Yonetani, 1976, 1977; Wikstrom *et al.*, 1976; Clore & Chance, 1978*b*; de Fonseca & Chance, 1978 since it provides an opportunity to study the mechanism of ligand binding in the absence of electron transfer. This reaction may be initiated by flash photolysis of the CO-enzyme complex under anaerobic conditions. The kinetics of the reaction between CO and solid-state fully reduced (*a*²⁺*a*₃²⁺) membrane-bound cytochrome oxidase over the 178–203K range have been analysed by non-linear optimization techniques (Clore & Chance, 1978*b*). Kinetic data were obtained by dual-wavelength multichannel spectroscopy at three wavelength pairs (591–630, 608–630 and 445–460nm), and small, but significant, differences were observed between the three normalized progress curves. On the basis of Beer's law, a minimum of three spectroscopically distinct species were needed to fit the data, and a two-intermediate sequential mechanism (Model 1) was found to fit the data best:

Thus flash photolysis of the CO-bound enzyme (I) yields free cytochrome oxidase (E) and CO, which then recombine to form a hypothetical intermediate (II). Intermediate II is then converted into the CO-enzyme complex (I) (identical with the compound existing before photolysis). A reinvestigation of this reaction (de Fonseca & Chance, 1978) revealed that when photolysis by the monitoring beam is taken

into account, model 1*a* (below) fails to fit the kinetic data, since some of the optimized parameters were badly determined. The data were best fitted by a single-intermediate mechanism (Model 2 below) (Chance, 1953) with an s.d. of 2%. However, the mean absolute correlation index ($\bar{c}$) of the statistical fit was significantly greater than unity ($\bar{c} = 2.5$). The corresponding reaction with half-reduced cytochrome oxidase (*a*³⁺*a*₃²⁺) gave a better fit to Model 2 ($\bar{c} = 1.3$).

We now present further optical data consistent with Model 2 and evidence of devitrification of the frozen solvent, which leads to spurious results.

Experimental

Biochemical methods

Bovine heart mitochondria were suspended in a medium containing 0.1M-mannitol, 0.03M-sucrose, 15mM-sodium phosphate buffer, pH 7.4, and 10mM-succinate and left for 10min at 298K to achieve anaerobiosis. CO [100% at 101325 Pa (1 atm)] is then bubbled in for 10min, ethylene glycol added (final concn. 30%, v/v) and the preparation resaturated with 100% CO. The final concentrations are CO, 1.2mM, and bovine heart mitochondria, 9mg/ml (containing 3 μM-cytochrome oxidase).

The fully reduced CO-saturated preparation is injected into a cuvette with a 2mm optical path and left for 10min at 298K to ensure anaerobiosis. It is then frozen at 195K and transferred to the Dewar flask of the spectrophotometer, which is maintained at the desired low temperature by a flow of thermoregulated N₂.

The half-reduced preparation is obtained by

equilibrating the fully reduced CO-saturated preparation with 1 mM-ferricyanide at 273 K for 1 min immediately before freezing at 195 K. Under these conditions, anaerobiosis is achieved, and the redox

potential is sufficiently high to maintain the enzyme in its half-reduced ($a^{3+}a_3^{2+}$) state.

The reaction is initiated by a liquid-dye laser flash (pulse width 5 μ s) which causes 85–90% photolysis.

Fig. 1. Absorption difference spectra of unliganded enzyme minus enzyme-CO complex after the flash photolysis of (a, b) fully reduced and (c, d) ferricyanide-pretreated enzyme-CO complex at 178 K

A change of gain occurred in the vicinity of 485 nm. The arrows indicate the wavelengths at which kinetic analyses were performed (see Fig. 2). A diffuser plate was used in obtaining (b) and (d). The experimental conditions are described in the text.

Fig. 2. Semi-logarithmic plots of the absorbance changes in the vicinity of 445, 590 and 610 nm in Figs. (a) 1a, (b) 1b, (c) 1c and (d) 1d

The time between successive scans was 95s. ---- in (a) indicates the slope of the continuous line of (b).

Biophysical techniques

Absorption difference spectra are obtained by using a Johnson Foundation dual-wavelength scanning spectrophotometer (Chance & Graham, 1971). The photomultiplier output is coupled to a 1024-bit digital spectral memory in which can be stored the characteristics of the baseline (the enzyme-CO complex, in this instance) and from which can subsequently be read out corrective signals to the

measuring wavelengths, effectively subtracting the stored baseline from the incoming data. The reference wavelength is 575 nm.

Optical effects due to changes in scattering properties of the sample (described in the Results section) are decreased by placing a diffuser plate (e.g. a strip of typewriter paper) between the sample and the photomultiplier. This acts as an extra scatterer and decreases the relative scattering change.

The kinetic data in Fig. 3 (below) are obtained by two time-sharing Johnson Foundation multichannel dual-wavelength spectrophotometers (Chance *et al.*, 1975 *a, b*), one affording wavelengths in the α -region and the other in the Soret region. The wavelengths are isolated by filters and are interlaced, one with another, at 8 ms intervals by synchronized 60 Hz rotating discs. The measuring beam is sufficiently intense to cause significant photolysis of the sample (de Fonseca & Chance, 1978). This prevents the progress curves (Fig. 3 below) from returning to the position before photolysis.

Results and Discussion

Fig. 1(a) shows the absorption difference spectra (unliganded enzyme minus enzyme-CO complex) at fixed time intervals after flash photolysis of the fully reduced enzyme-CO complex, the first scan being obtained immediately after photolysis at 178 K. A time-dependent increase in the light-scattering properties of the frozen sample, presumably due to devitrification (Keilin & Hartree, 1950), caused an apparent increase in absorbance at shorter, more scattered wavelengths, and an apparent decrease at longer wavelengths, resulting in a clockwise rotation of the entire spectrum about the reference wavelength (575 nm). Fig. 2(a) shows a semilogarithmic plot of the normalized absorbance changes at 445 nm, 590 nm and 612 nm. The non-linear nature of the change at 612 nm can be partly corrected for using the absorbance changes at a longer wavelength (638 nm) and Rayleigh's law. According to Rayleigh's law, the ratio of the scattering change between 612 and 638 nm (with 575 nm as the reference) is given by $(575^{-4} - 612^{-4}) / (575^{-4} - 638^{-4})$, i.e. 0.65. Hence the corrected absorbance change at 612 nm is $\Delta A_{612} - 0.65(\Delta A_{638})$. Scattering changes such as those in Fig. 1(a) are less conspicuous at higher temperatures (when recombination rates are faster) and higher concentrations (when the total absorbance change due to photolysis is larger).

Fig. 1(b) shows spectra obtained with a diffuser plate between the identical sample and photomultiplier. The corresponding absorbance changes shown in Fig. 2(b) are linear and identical at all three wavelengths studied, consistent with Model 2. No initial phase of the absorbance change can be detected at temperatures as low as 157 K, when the recombination rate is negligible (Chance *et al.*, 1965).

The small differences between the progress curves observed at the three wavelength pairs by multichannel dual-wavelength spectroscopy (Clare & Chance, 1978b), especially at the lower temperatures, may be decreased considerably by using a diffuser plate. Fig. 3 shows such data at 183 K on the recombination of CO with fully reduced membrane-bound cytochrome oxidase. The curves are identical

Fig. 3. Normalized absorbance changes at (○) 445–460 nm, (□) 630–591 nm and (△) 608–630 nm for the reaction of fully reduced cytochrome oxidase with CO at 183 K.

The experimental conditions are similar to those in Fig. 1(b).

within experimental limits, and fit Model 2 ($\bar{c} = 1.5$; s.d. of fit = 2%) with good determination of all the optimized parameters (relative s.d. < 5%).

Figs. 1(c) and 1(d) show absorption difference spectra of the reaction of half-reduced ($a^{3+}a_3^{2+}$), cytochrome oxidase with CO, in the absence and presence, respectively, of a diffuser plate. Figs. 2(c) and 2(d) show the corresponding semilogarithmic plots of the absorbance changes. In this case, identical linear plots are obtained at the three wavelengths, even in the absence of the diffuser plate. It is noted that the isosbestic points are better defined in the presence of a diffuser plate (Fig. 2d). Ferricyanide pretreatment has very slightly blue-shifted the peak and trough in the α -region, and decreased the rate of recombination. Such experiments have repeatedly shown that the amplitude of the absorbance change does not change significantly on ferricyanide pretreatment.

Conclusion

Optical data obtained on highly scattering frozen samples may include information about devitrification and/or vitrification that would confound kinetic analyses unless proper precautions are observed. The reliability of such optical data is dependent on the monitoring and reference wavelengths and on the magnitude and the rate of change of the absorbance change. As an illustrative example, when observations are made at 591–630 nm (or 575–612 nm), intermediates whose half-times of formation are greater than 1 min and whose contribution to the total absorbance change is less than 0.02 ΔA and for which there are no distinctive absorbance maxima should be accepted only with great caution (Clare & Chance, 1978a, b). Our studies show only one spectroscopic form of carbonmonoxide-cytochrome oxidase.

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