THE USE OF PRINCIPAL COMPONENT ANALYSIS TO RESOLVE THE SPECTRA AND KINETICS OF CYTOCHROME *c* OXIDASE REDUCTION BY 5,10-DIHYDRO-5-METHYL PHENAZINE

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ABSTRACT The method of principal component analysis (PCA) was applied to the absorption-wavelength-time surfaces generated by rapid scanning stopped-flow spectrophotometry (RSSFS). The method was used to resolve the absorption surfaces generated during the reduction of cytochrome *c* oxidase by 5,10-dihydro-5-methyl phenazine (MPH) into the individual spectral shapes and time courses of the component chromophores. Two forms of resting cytochrome oxidase were used in these analyses: one that has its maximum absorption in the Soret region at 418 nm (418-nm species) and the other has its absorption maximum at 424 nm (424-nm species). A weighting scheme suitable for RSSFS data was developed. The optical absorption spectra obtained by W.H. Vanneste (1966, *Biochemistry*, 5:838–848) for the oxidase components were found to fit adequately as components of the experimental surfaces. Among these spectra were the oxidized forms of cytochromes *a* and a_3 in the wavelength region 330–520 nm for the 418-nm species. Vanneste's spectral shape for the oxidized cytochrome a_3 did not fit as a component in the spectrum of the 424-nm species. After accounting for the spectral shape of all components present, PCA provided a straightforward method for determining the separate time courses of each chromophore. We have found for both forms used that cytochrome *a* is reduced by MPH in the initial stages of the reaction, while cytochrome a_3 is reduced in subsequent, slow phases. An important aspect of PCA is that it provided confirmation of the spectra of the various oxidase components without requiring the use of inhibitors or the use of simplifying mechanistic assumptions. The resolution of time profiles of strongly overlapping chromophores is also demonstrated.

INTRODUCTION

Optical absorption spectroscopy has been of fundamental importance in the characterization of cytochrome *c* oxidase (Lemberg, 1969). The protein spectrum has peaks in the near ultraviolet (UV), visible, and near infrared (IR) regions that are sensitive to the oxidation and ligation states of its four metal centers. The absorption spectra of cytochromes *a* and a_3 of the oxidase overlap strongly in the Soret and α-band regions. Also, in these two regions absorbance changes due to the oxidase copper centers may be obscured because of the relatively small extinction coefficients expected for copper complexes compared with those of the heme moieties. Nonetheless, and despite recent controversies, the classical aa_3 picture of the oxidase, including assignments of wavelength maxima and extinction coefficients for the two hemes (Yonetani, 1960; Horie and Morrison, 1963; Vanneste, 1966) has been rather widely accepted (for reviews, see Malmstrom, 1979, and Wikstrom et al., 1981). It has been argued (Caughey et al., 1976), however, that it is unwise to synthesize spectra of cytochromes *a* and a_3 on the assumption that the properties of one heme are not affected by changes in the oxidation or ligation states of the other metal components as was done in the inhibitor studies. The basis for this criticism is the strong evidence now available for electronic and magnetic interactions between the metal components of the oxidase (Hartzell et al., 1973, 1974; Babcock et al., 1978, Blair et al., 1982).

For the past few years, we have been using rapid scanning stopped-flow spectrophotometry (RSSFS) for the study of the electron transfer kinetics of cytochrome oxidase (Halaka et al., 1981, 1984). In RSSFS, a particular region of the absorption spectrum of the compounds under study is rapidly and repeatedly scanned. The result is an absorption surface that is a function of time and wavelength. During the course of these studies, we noticed a complexity in the temporal characteristics of these surfaces in the Soret and the α bands. It was qualitatively possible to distinguish between cytochromes *a* and a_3 on the basis of the time dependence of the spectra during the reduction with 5, 10-dihydro-5-methyl phenazine (MPH).

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The results indicated that MPH interacts with the *a* site of the oxidase (Halaka et al., 1981, 1984). Other reductants, for example, hexaaquochromium(II) (Greenwood et al., 1977) and hexaammineruthenium(II) (Scott and Gray, 1980), have also been used in reduction studies of the enzyme. Although similar time-resolved optical difference spectra were constructed in these two studies and in our own earlier work, the absolute spectral shapes of the overlapping cytochromes *a* and a_3 were difficult to resolve as were the detailed time courses of the individual chromophores.

The method of principal component analysis (PCA), also known as principal factor analysis (PFA), has been suggested for the study of interacting components. With matrix manipulation techniques PCA can, in favorable cases, resolve complicated surfaces such as those described in this paper. The important information obtainable from the data described here (and, indeed, from data obtained by other techniques in which an observable is measured as a function of two independent variables) are (*a*) the minimum number of components that can describe the behavior of the system, and (*b*) the contribution of each component to each of the variable domains. In the case of RSSFS, one is interested in determining the number of chromophores, their spectral shapes, and their time courses.

Here we apply PCA to the data obtained from RSSFS. Besides the importance of the answers to the classical aa_3 picture of cytochrome *c*-oxidase, our intention is to also demonstrate the general applicability of the method to complicated systems.

METHOD OF PRINCIPAL COMPONENT ANALYSIS

Data collected as a function of two independent variables can be written as a matrix in which the rows describe the dependence on one variable and the columns the other variable. PCA has been applied to data obtained by a variety of physical techniques (Blumer and Shurvell, 1973; Sylvestre et al., 1974, Vadaski, 1974, Ritter et al., 1976; Morse and Chan, 1980; Shrager and Hendler, 1982; Hofrichter et al., 1983). As the mathematical formulation of the method has been described previously (Anderson, 1963; Cochran, 1977; Cochran and Horne, 1977, 1980; Cochran et al., 1980), only the application of PCA to RSSFS data will be considered here. A brief outline of the method is presented below for the sake of clarity. We also present a new weighting scheme that, although specific to RSSFS data, can be modified to apply to other physicochemical methods.

Note that from the outset there are no mechanistic assumptions needed for the application of PCA to kinetics data. We only require that the absorbance at every wavelength be a linear function of the concentration of the absorbing species (Beer's law). If the data are collected at p wavelength channels N times, the resulting matrix **A**, is of order pXN. The element A_{ij} of this matrix (absorbance at wavelength i and at time j) is represented as

$$A_{ij} = \sum_{k=1}^{q} f_{ik} c_{kj}, \tag{1}$$

where f_{ik} is the molar absorptivity (times the pathlength of the absorbance cell) of absorber k at wavelength channel i, c_{kj} is the molarity of k at the time corresponding to scan j, and q is the number of absorbers. Accordingly, the matrix **A** can be factored into two matrices

$$\mathbf{A} = \mathbf{F}\mathbf{C}^{\mathrm{T}}. \tag{2}$$

The **F** matrix describes the spectral shapes of the absorbers while the **C** matrix contains information on the concentration as a function of time. **F** is a $(p \times q)$ matrix defined by $\mathbf{F} = (\mathbf{f}_1, \mathbf{f}_2, \ldots \mathbf{f}_q)$. The vector $\mathbf{f}_k$, called the static spectrum of absorber k, is a **p** component column vector whose ith element is the product of the cell pathlength and the molar absorptivity of absorber k at wavelength channel i. **C** is defined by $\mathbf{C} = (\mathbf{c}_1, \mathbf{c}_2 \ldots \mathbf{c}_q)$, where $\mathbf{c}_k$, the concentration vector of absorber k, is an N component column vector whose jth element is the molarity of absorber k at the time at which scan j was made. The ultimate goal of PCA application is the determination of the number of independent components q, as well as the columns of both **F** and **C**.

WEIGHTS FOR PRINCIPAL COMPONENT ANALYSIS

Experimental errors must be considered in order for PCA to give correct results. Improper weights can lead to erroneous estimates of the number of absorbing species (Cochran, 1977; Cochran and Horne, 1977). When errors are included, the experimental matrix **A** becomes

$$\mathbf{A} = \mathbf{F}\,\mathbf{C}^{\mathrm{T}} + \epsilon \tag{3}$$

in which the matrix ϵ contains the experimental errors whose estimation for RSSFS data will now be described. Random errors in absorbance measurements obtained by our scanning stopped-flow apparatus can be traced to two factors: (*a*) a time-dependent factor that is minimized by signal averaging, and (*b*) a position-dependent component that depends on the position of a channel in the spectrum as well as its nominal absorbance. When it is possible to separate errors into time and position contributions, the variance (error matrix element ϵ_{ij}) can be written as

$$(\epsilon_{ij}) = X_i Z_j, \tag{4}$$

where Z_j is a function of the spectrum number (time) and X_i is a function of the wavelength channel number. This model for variance leads to a statistically weighted data matrix $\mathbf{A}_w$, defined as

$$\mathbf{A}_w = \mathbf{L}\,\mathbf{A}\,\mathbf{T}, \tag{5}$$

where **L** and **T** are diagonal matrices whose elements are the X_i's and Z_j's, respectively (Eq. 4).

Z_j depends on the nature of the on-line averaging procedure and becomes smaller at longer times of data collection when averaging is performed (Cochran et al., 1980). The correct wavelength-dependent weighting factor X_i should account for three major noise contributions to the absorbance at channel i: (*a*) the wavelength corresponding to this channel (noise depends on the lamp output and photomultiplier tube response); (*b*) the magnitude of the absorbance, and (*c*) the rate of change of absorbance with wavelength at the particular channel being considered. The third contribution originates from errors in the precise wavelength reproducibility at each channel caused by mechanical vibrations of the mirror system used (Papadakis et al., 1975; Coolen, 1975; Coolen et al., 1975). Simply, this means that if a chromophore has a sharp peak, the sides of the peak will have the largest measurement errors. Hence, a weighting procedure that includes spectral peaks similar to those observed in the stopped-flow experiment is a distinct advantage.

Values of X_i for each wavelength channel in the present work were obtained by averaging the results of two weight experiments, which covered the same wavelength region as for the reaction under study. In one experiment the spectrum of the enzyme mixed with buffer was obtained while for the other weight experiment the final product spectrum was obtained. These spectra were time independent and an average weight was calculated from the standard deviations at each wavelength channel. This method should be a good approximation for reactions that do not produce intermediates with spectral peaks that are greatly different from those of the reactants or products. With slight modifications, this weighting method can be used for other physical systems to which PCA is applicable.

DETERMINATION OF NUMBER OF COMPONENTS

Cochran and Horne (1977) have shown that two kinds of PCA are useful for kinetics experiments; each requires only the matrix $\mathbf{A}_w$ and each gives an estimate of the number of components, q. Second moment matrix PCA, called M analysis, gives the minimum number of independent absorbers (chromophores) required to interpret the experiment. The matrix $\mathbf{M}_w$ is defined by

$$\mathbf{M}_w = (1/N)\mathbf{A}_w\mathbf{A}_w^T. \tag{6}$$

Sample covariance matrix PCA, or S analysis, gives the minimum number of absorbers whose concentrations change independently during the experiment. $\mathbf{S}_w$, which is essentially a matrix of difference spectra, is defined by

$$\mathbf{S}_w = [1/(N-1)](\mathbf{A}_w - \bar{\mathbf{A}}_w)(\mathbf{A}_w - \bar{\mathbf{A}}_w)^T, \tag{7}$$

where the mean absorbance at wavelength channel i is given by

$$A_i = (1/N)\sum_{j=1}^{N} A_{ij}. \tag{8}$$

The M and S analysis estimates of q are not necessarily the same. The rank of **M**, m, is the same as that of **A** and the rank of **S** is the same as that of $(\mathbf{A} - \mathbf{A}_w)$ (Bellman, 1970). Details and rules for the ranks of **M** and **S** are discussed by Cochran and Horne (1977).

The rank of **M** gives an estimate of the number of detectable absorbing components. This may be less than the actual number of components for several reasons including the following (*a*) Multiple absorbers that do not change with time contribute only a single component to PCA. (*b*) Nonabsorbing species or those whose extinction coefficients are too small are not detected and so cannot contribute to PCA. (*c*) If the concentration of one absorber is always directly proportional to that of another absorber, then both will appear together as a single component in PCA. On the other hand, a single molecular species with two independent chromophores that change differently with time will appear as two components in PCA. For example, the a and a_3 chromophores of cytochrome c oxidase appear as separate components in PCA because they have different time courses.

Having constructed $\mathbf{M}_w$ and $\mathbf{S}_w$, the number of components (rank) can be determined by diagonalizing the corresponding matrix. In principal, the number of nonzero diagonal elements (eigenvalues) is equal to the number of independent components present. However, due to experimental error, generally all the diagonal elements are nonzero. A practical approach toward determining q can be found in the following.

(*a*) The reconstructed absorbance matrix, $\hat{\mathbf{A}}(r)$ is calculated by using 1, 2, . . . r components and compared with the experimental matrix. The definition of $\hat{\mathbf{A}}(r)$ is given by Cochran and Horne (1977). It is essentially the best fit that can be made to the absorbance surface by using r chromophores. For a noise-free experiment $\hat{\mathbf{A}}(r) = \mathbf{A}$ when $r = q$. In the presence of noise, one may conclude that the number of components is the same as r when the difference between the two matrices is of the order of experimental error. A graphical representation of the three-dimensional residual surface $[\hat{\mathbf{A}}(r) - \mathbf{A})]$ is usually helpful in determining when r is equal to m. When $r = m$ this surface should be random if the errors are random.

(*b*) With statistically weighted PCA, when $r = m$, the value of the function $Q_r/[(N-r)(p-r)]$ is approximately unity where Q_r is given by Eq. 9 (Cochran, 1977). (This is equivalent to Eq. 31 of Cochran and Horne, 1977.)

$$Q_r = \sum_{i=1}^{p}\sum_{j=1}^{N} L_i T_j [\hat{A}_{ij}(r) - A_{ij}]^2. \tag{9}$$

By using both of the above methods it is easy to determine the number of major contributors to the absorbance-

wavelength-time surface. Note, however, that systematic time-dependent errors can interfere with minor contributors and that the sum of contributions of absorbers with nearly the same spectra can appear as a single absorber.

TARGET TESTING BY PCA

PCA provides a means by which to test whether a proposed spectral shape (or a concentration-time profile) fits as one of the column vectors of the **F** or **C** matrix (Eq. 2). This is called "target testing" (Malinowski and Howery, 1980). It can be shown (Cochran et al., 1980) that a proposed spectral shape function or concentration-time profile can be tested by means of a least-squares criterion to see whether it is acceptable as a contribution from one of the absorbers. Furthermore, the proposed function or profile need not span the entire region of wavelength or time. If the fit is satisfactory over a limited (but significant) region, then from the entire absorbance-wavelength-time surface one can estimate the behavior of this absorber outside the range of the trial function or time profile. If the spectral shape functions, $\mathbf{f}_k$, are known for all m components, then all of the concentration-time profiles, $\mathbf{c}_i$, can be determined and vice-versa. Once again, we emphasize that this procedure does not require any information about the mechanism. However, it is possible to test the concentration-time profiles predicted by a particular mechanism. If the fit to the data is satisfactory, then the spectral shapes of all components can be determined. Of particular interest to the present study is the ability to predict the spectral shapes of absorbers in regions of strong band overlap from their shapes in regions of good resolution. The application of these methods of resolution of the spectral shapes and concentration-time profiles of cytochrome *c* oxidase during reduction by MPH is the subject of the remainder of this paper.

MATERIALS

Cytochrome oxidase was prepared as described by Hartzell and Beinert (1974); the details of the handling of the enzyme and of the apparatus used in recording the stopped flow, rapid scan data are given elsewhere (Halaka et al., 1981, 1984). Principal component analysis was carried out in detail on data obtained in two separate studies with two different forms of the oxidized protein (for biochemical details [Halaka et al., 1984]): the resting enzyme with a Soret absorption maximum as isolated at 418 nm (designated the 418-nm species) and the resting enzyme with a Soret absorption maximum as isolated at 424 nm (designated as the 424-nm species). Applying PCA to these forms complements our previous work on the kinetics of the anaerobic four-electron reduction of the oxidase (Halaka et al., 1981). It also addresses the question of the nature of the spectral shift in the cytochrome a_3 spectrum in the Soret region and its relation to activity of electron transfer of the oxidase. In both cases, the spectra of cytochromes a and a_3 strongly overlap in both the Soret and the α bands. Assigning full time courses for each of the cytochromes in the native protein was the major impetus for applying PCA to these forms. Two wavelength regions were used in the RSSFS experiments described (see below) to optimize resolution of particular spectral shapes. The previously reported rate data (Halaka et al., 1981) were obtained on the same instrument in the fixed wavelength mode. Although one could also use PCA to determine rate constants, we chose not to do so for two

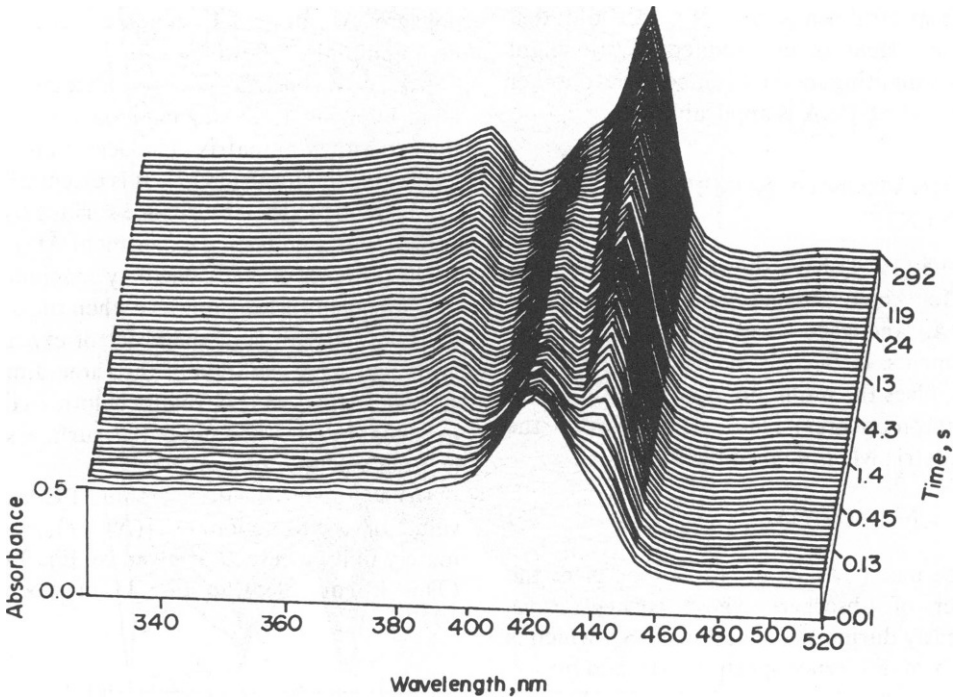

FIGURE 1 Experimental absorbance-wavelength-time surface for the anaerobic reduction of 3.8 μM cytochrome oxidase (418-nm species) by 8.4 μM MPH. The data consisted of 60 wavelength channels and 56 scans. The medium was 50 mM HEPES containing 0.5% Tween 20, pH = 7.4. Cell path length = 1.85 cm; T = 21°C.

reasons. (*a*) The time between successive scans (13.3 ms) is long enough that short-time information is sparse. In the fixed wavelength mode this is not a problem. (*b*) Additional noise caused by rotation of the monochromator mirror is introduced in the scanning experiments. Thus, our strategy is to use the rapid-scan information with PCA to identify spectra and determine the time courses of each chromophore and to use fixed wavelength data to determine rate constants.

RESULTS AND DISCUSSION

Reduction of the Resting Enzyme (418-nm species) by MPH

In the wavelength region from 330–520 nm, the data contain information on the spectral shapes in the Soret region (Halaka et al., 1981) and kinetic profiles of the reduced and oxidized cytochromes *a* and a_3 of the oxidase as well as those of the MPMS/MPH couple (Halaka et al., 1982). We describe here the steps taken to resolve the independent components in this region.

Number of Absorbers

We emphasize again that the term absorber may in fact refer to several species whose concentrations are always proportional to each other (in M analysis) or whose rates of growth and/or decay are proportional (in S analysis) or whose spectral shapes are very similar (in both M and S analysis). Fig. 1 presents the wavelength-time-absorbance experimental surface in the Soret region obtained on mixing 3.8 μM cytochrome *c* oxidase anaerobically with 8.4 μM MPH. Principal component analysis surfaces were reconstructed for r values from 1 to 5. Visual comparisons showed that the reconstructed surface for the case of three absorbers qualitatively reproduced the experimental surface.

The residual surface $[\hat{\mathbf{A}}(r) - \mathbf{A}]$, which should be random at $r = m$, confirms the assignment of $r = 3$. In Fig. 2 *a*, the residual surface is shown for $r = 3$. The residuals constructed by using only two eigenvectors are shown in Fig. 2 *b*. The residuals shown in Fig. 2 *a* are small and fluctuate around zero, except for several large noise peaks on the sides of the Soret absorption band (which are caused by mirror vibrations). The residuals shown in Fig. 2 *b* have large systematic deviations. Another piece of evidence for the assignment $r = 3$ is that the values of the function $Q_r/(N - r)(p - r)$ are 722, 12, and 2.1 for $r = 1$, 2, and 3, respectively. The assignment $r = 3$ was obtained for two

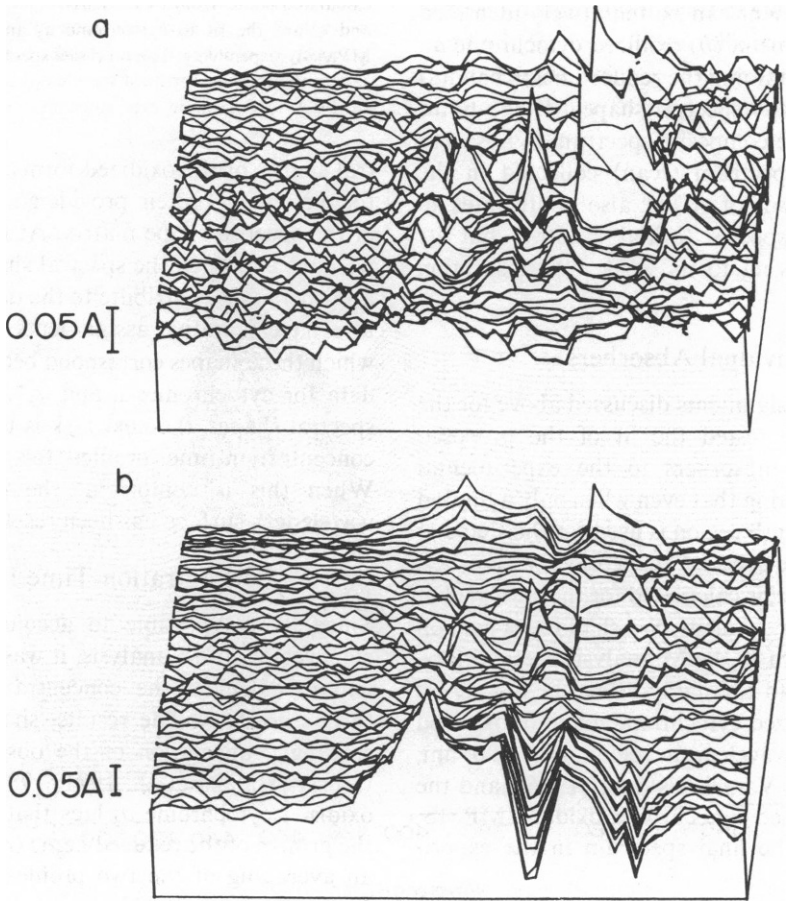

FIGURE 2 (*a*) Residual surface $[\hat{\mathbf{A}}_{(3)} - \mathbf{A}]$, obtained by subtracting the data in Fig. 1 from the reconstructed matrix $\hat{\mathbf{A}}_{(3)}$ in which three eigenvectors were used ($r = 3$) in M analysis; A is the experimental surface. (*b*) Residual surface $(\hat{\mathbf{A}}_{(2)} - \mathbf{A})$ for the case of two eigenvectors ($r = 2$) in M analysis.

separate rapid-scan experiments for this wavelength region.

Having determined the minimum number of components that can describe the experimental data surface, the next step is to account for the elements of either the spectral shape maxtrix (**F**) or the concentration-time (**C** matrix) profiles. We have chosen to try to account for the elements of **F**, since proposed spectral shapes have been assigned to the oxidase components from ligand-binding studies (Vanneste, 1966). There are six predicted chromophores in this wavelength region. These are the cytochromes a^{3+}, a^{2+}, a_3^{3+}, a_3^{2+}, 5-methylphenazinium methyl sulfate (MPMS), and MPH. The absorbance changes due to MPH oxidation to MPMS are coupled to the reduction of the two cytochromes since the concentrations were close to the stoichiometric ratio (2 MPH: 1 cytochrome oxidase [Halaka et al., 1981]). Because of the strong overlap of the a and a_3 bands in the Soret region for the reduced enzyme, the limited resolution, and the fact that the region of the α band was not included in this experiment, the two reduced heme bands could not be separated, although they could be easily resolved when the longer wavelength region was included (see below).

Based upon these considerations, three independent absorbers in this experiment can be tentatively identified as (*a*) oxidized cytochrome *a*, (*b*) oxidized cytochrome a_3, and (*c*) the spectrum containing the reduced heme band(s) and the MPMS (oxidized) spectral shapes. If the latter assignment is correct, the composite spectrum of absorber 3 is actually the last spectrum (scan) collected in the experiment. The spectrum of MPH also contributes to those of the oxidized species (initial spectra) but its contribution is small owing to its weak UV absorption (Halaka et al., 1982).

Spectra of Individual Absorbers

By using the tentative assignments discussed above for the suspected absorbers, we tested the fit of the proposed target spectra of these absorbers to the experimental surface. It is worth repeating that even when only a limited (but significant) wavelength region is used for the shape of a suspected absorber, the spectral shape of that absorber, which best fits the surface, is calculated for the entire set of experimental wavelength channels. We shall focus here on the fit of proposed spectra to the M analysis eigenvectors. As target spectra to fit to the data surface in Fig. 1, we used the spectra of oxidized cytochrome *a* and of oxidized cytochrome a_3 in the wavelength region 400–490 nm, which we obtained from Vanneste's data (1966), and the spectrum of fully reduced cytochrome oxidase/MPMS, which we obtained as the final spectrum in the experiment.

The M-analysis fit of the proposed spectral shapes of the oxidized cytochromes *a*, a_3, and of the reduced oxidase (+MPMS contribution) are shown in Fig. 3. It is clear that Vanneste's assignments (Vanneste, 1966) of the spectral shapes of the oxidized forms of cytochromes *a* and a_3 for the resting protein provide good fits to the eigenvectors of the spectral shape matrix. At this stage of the analysis we have extracted the spectral shapes for the independent absorbers that contribute to the data surface of Fig. 1. We also have tentative assignments of the chromophores to which these shapes correspond because we used Vanneste's data for cytochromes *a* and a_3^{3+}. Having determined the spectral shapes, the next task is to use them to obtain the concentration-time profiles for each of the absorbers. When this is completed, the entire absorbance-time-wavelength surface has been resolved.

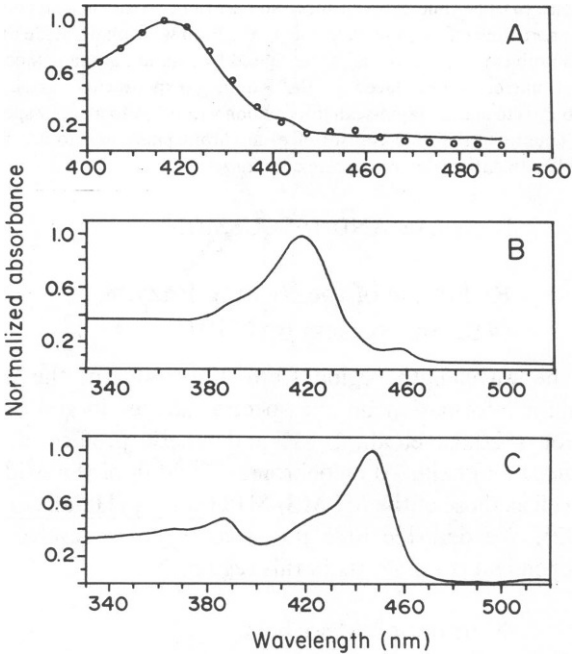

FIGURE 3 (*a*) M-analysis fit of the target oxidized cytochrome spectrum to the data presented in Fig. 1. The solid line represents the proposed spectrum (from Vanneste, 1966) and the circles are the calculated points from PCA. Three eigenvectors were used for this fit. *B* and *C* are the fit to cytochrome a_3 and to the reduced oxidase (+ MPMS), respectively. The proposed spectra and the fit are indistinguishable in these cases.

Concentration-Time Profiles

Since it was possible to account for all the m (= 3) eigenvectors in M analysis, it was a straightforward problem to calculate the concentration-time profiles of the three absorbers. The results, shown in Fig. 4, provide a striking confirmation of the observations described previously (Halaka et al., 1981, 1984), that the decay of the oxidized cytochrome a_3 lags that of the oxidized *a*. Also, the growth of the reduced heme ($a + a_3$) band results from an averaging of the two profiles. Without the results of PCA we were only able to conclude that the reduction of cytochrome *a* is faster than that of a_3. Now we can conclude that the fast phase consists exclusively of the reduction of cytochrome *a* and that the slow phases

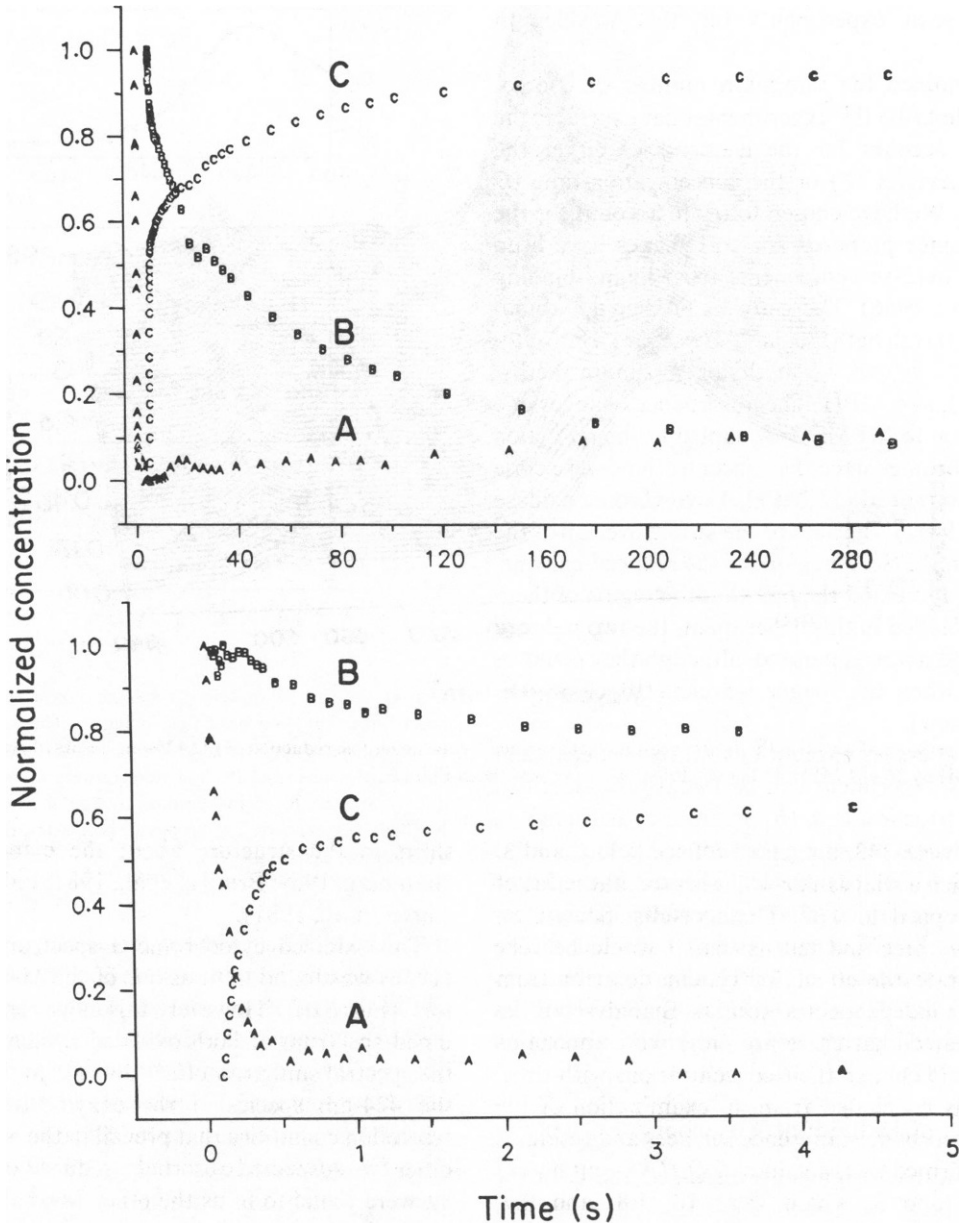

FIGURE 4 Concentration-time profile for the disappearance of oxidized cytochrome *a* (*A*), oxidized cytochrome a_3 (*B*), and the growth of the combined reduced peak (*C*). Concentrations of each species are normalized. Bottom figure shows an expanded view of the first 5 s of the reaction.

represent the slower reduction of cytochrome a_3. Thus cytochrome *a* is the site of reduction by MPH; the slower reduction of cytochrome a_3 is, most likely, the result of an intramolecular electron transfer (Halaka et al., 1981, 1984). Although this was anticipated from our earlier results, PCA gives an objective confirmation of the time courses of the two chromophores and, in addition, provides their resolved optical spectra.

Reduction of the Resting Enzyme (424-nm Species) by MPH

Here we use the data surface to determine the contribution of both reduced cytochromes *a* and a_3 to the α band of the reduced protein as well as to its Soret absorbance. In this wavelength region (400–650 nm) the MPMS/MPH couple does not contribute appreciably to the absorbance. The experimental absorbance-wavelength-time data surface is shown in Fig. 5 for the reduction of 3.3 μM cytochrome oxidase by 26 μM MPH.

Number of Components

M analysis gave for the rank of M a value of 3. This was concluded from a comparison of the reconstructed absorbance surface for $r = 2$ and $r = 3$ with the experimental surface and by examination of the residual surfaces for $r = 2$ and $r = 3$. Also, the values of the function Q_r/

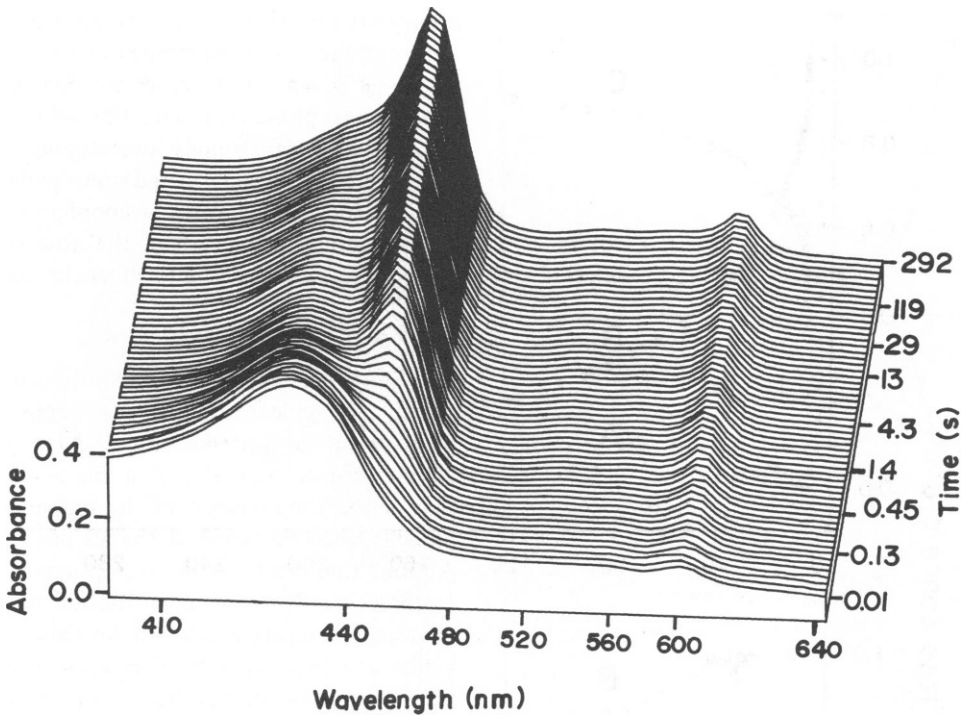

FIGURE 5 Experimental wavelength-absorbance-time data surface for the anaerobic reduction of the 424-nm species of resting cytochrome oxidase (3.3 μM) by 26 μM MPH. Other conditions were the same as in Fig. 1.

$(N - r)(p - r)$ were 343, 8.4, and 1.6 for r = 1, 2, and 3, respectively. When a trial rank r = 4 was used, the value of this function dropped to 0.67. These results indicate an essential rank of three and tell us that it would be very difficult to separate the effects of systematic error from those due to four independent absorbers. S analysis of the same data indicated that there are only two components that independently change their concentrations with time, r_s = 2. This was concluded from an examination of the reconstructed absorbance difference surfaces and residuals and further confirmed by the values of $Q_r/(N - r)(p - r)$ for r_s = 1, 2, and 3, which were 18, 1.8, and 0.7, respectively. A value of r_s smaller by one than r_m is common when both reactants and products absorb since the rates of production of products are proportional to the rates of disappearance of reactants.

Spectra of Individual Absorbers

There are four plausible target absorbers for fitting the eigenvectors in this case (M analysis). These are the oxidized and reduced cytochromes a and a_3. Unlike the case discussed for the shorter wavelength experiments (Fig. 1), the fit of the oxidized cytochrome a_3 spectrum from Vanneste (1966) to the data was unsatisfactory. This is explainable, however, by postulating that cytochrome a_3^{3+} in the 424-nm species of the enzyme has its peak shifted closer to that of cytochrome a^{3+}. This is consistent with previous work that indicates that the variation in spectral properties of the oxidized enzyme results from shifts in the structure about the cytochromes a_3^{3+} site (Lemberg, 1969; Brudvig et al., 1981; Babcock et al., 1981; Carter et al., 1981).

The oxidized cytochrome a spectrum from Vanneste (1966) was found to fit as one of the M-analysis eigenvectors (Fig. 6 *a*)]. However, this now represents the combined spectrum of both oxidized cytochromes because of the spectral similarity of a^{3+} and a_3^{3+} in the Soret region in the 424-nm species of the enzyme and from the low resolution conditions that prevail in the α band region. The other two suspected absorbers, reduced cytochromes a and a_3, were found to fit as the other M-analysis eigenvectors. The reduced forms of the two cytochromes were resolvable in this case because of better wavelength resolution in the Soret region, as well as different contributions to the α band. It is apparent that reduced cytochrome a (Fig. 6 *b*) has a much higher absorption at ~600 nm than does reduced cytochrome a_3 (Fig. 6 *c*). This has been suspected for some time, but the present data provide further experimental proof that the contribution of cytochrome a in this region strongly predominates. There is also a pronounced shoulder at ~425 nm in the spectrum of reduced cytochrome a_3. This result, postulated by Vanneste (1966), is clearly verified in this work. Note that this method can identify one absorber's spectrum over a wide wavelength range. The absorber's contribution to two peaks can be estimated. This is due to the fact that a single component will have the same time course over its whole spectrum. The results shown in Fig. 6 for the absorbance in the α-band region might suggest a lower absorbance at ~600

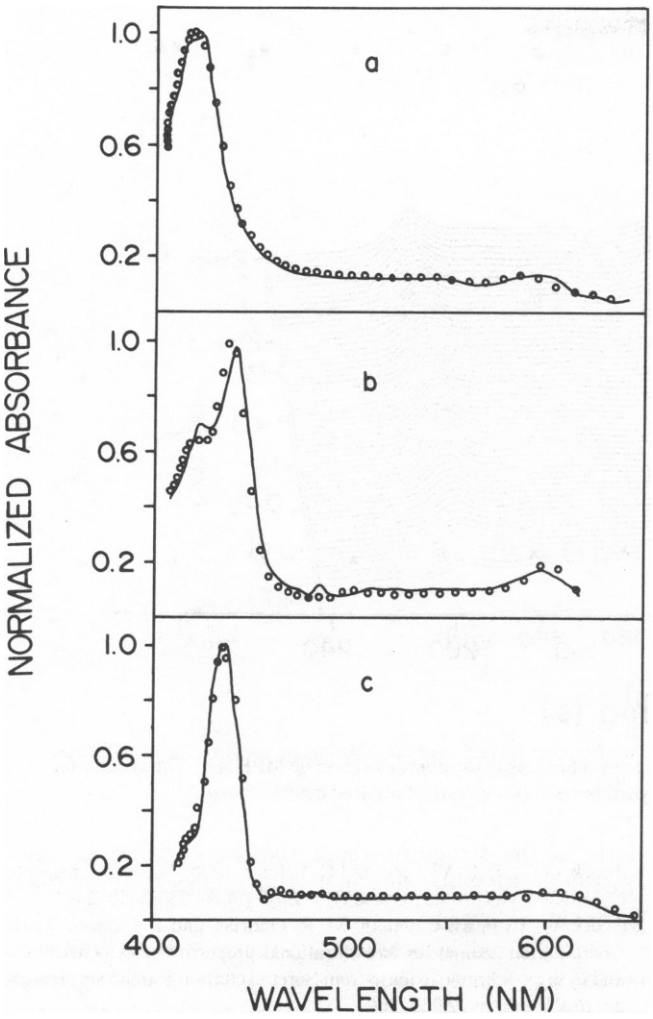

FIGURE 6 (*a*) Three eigenvector M-analysis fit to the spectrum of oxidized *a* and a_3 for the data surface of Fig. 5. (*b*) M-analysis fit of the reduced cytochrome *a* spectrum. (*c*) M-analysis fit to the spectrum of reduced cytochrome a_3. For all three panels the solid lines represent the proposed spectra (Vanneste, 1966) and the circles are the calculated, least-squares fit to the data surfaces (Fig. 5).

nm than is actually the case. This is the result of the low resolution (~8 nm/channel) because of the wide scan range used in this experiment. The α-band is rather sharp and it is artificially broadened and reduced in amplitude in these experiments. If a narrower scan range were used, this problem would be eliminated.

Concentration-Time Profiles

The concentration-time profiles of the three absorbers (Fig. 7) give excellent confirmation of the conclusions reached by studying the shorter wavelength region (Fig. 4). Clearly, cytochrome a_3^{3+} reduction lags the reduction of cytochrome a^{3+}, once again confirming that MPH reduces a^{3+} first. Note that in this wavelength region, we have the concentration-time profile for the appearance of cytochromes a^{2+} and a_3^{2+} (from which we have inferred the behavior of their respective oxidized forms), which can be compared with those of the disappearance of the oxidized forms of the two cytochromes in the shorter wavelength region (Fig. 4). Once again, we conclude unequivocally that the fast phase represents the reduction of cytochrome a^{3+}. In spite of strongly overlapping bands, PCA has provided both wavelength and time resolution of the contributions from the separate chromophores. A real advantage of the method is that all of the absorbance-wavelength-time data points are used to effect this resolution.

CONCLUSIONS

The work presented here illustrates the applicability of principal component analysis as a means to resolve strongly overlapping spectral shapes that have different temporal characteristics. As is shown in this work, complete resolution of the time courses of the individual components is possible once the spectral shapes have been determined. Although no mechanistic assumptions were used to arrive at these time profiles, confirmation of proposed mechanisms is obviously simplified by these techniques. PCA, which uses least-squares criteria for fitting of target (suspected) spectral shapes, has also provided a confirmation of the spectral shapes of cytochromes *a* and a_3 of the oxidase. Previous studies (Vanneste, 1966), in which these aspects of cytochrome oxidase were addressed, relied on differential inhibitor binding or carbon monoxide photochemical action spectra and thus were susceptible to ambiguities introduced by these added reagents (Caughey et al., 1976). That results similar to this earlier work were obtained by our PCA approach, in conjunction with recent interpretations of the potentiometric behavior of cytochromes *a* and a_3 (Babcock et al., 1978; Wikstrom et al, 1976; Carithers and Palmer, 1981), lends additional support to the neoclassical view of cytochrome oxidase (Nicholls and Chance, 1974; Wikstrom et al., 1976, 1981; Carithers and Palmer, 1981).

The spectra predicted by PCA depend somewhat, but not strongly, on the target spectra used. Thus minor changes in the target spectra have essentially no effect on the spectra obtained from the data surface by PCA. If, however, the target spectra are greatly in error, the resultant spectra from PCA are also in error since this method finds the best least-squares fit of the target spectra to the surface.

The concentration-time profiles of the heme *a* components of cytochrome oxidase, which were deduced from PCA (Figs. 4 and 7), agree well with our previous conclusions for the reduction of the enzyme by MPH. We have assigned cytochrome a^{3+} to be the primary reduction site by MPH (Halaka et al., 1981, 1984). This conclusion was based on different kinetic activity of the two chromophores and on difference spectral shapes during the reduction. PCA not only confirmed this observation, but indicated a clear separation between the time courses of the reduction of a^{3+} and a_3^{3+}. In the two different resting forms of the enzyme that we have examined, we found the same

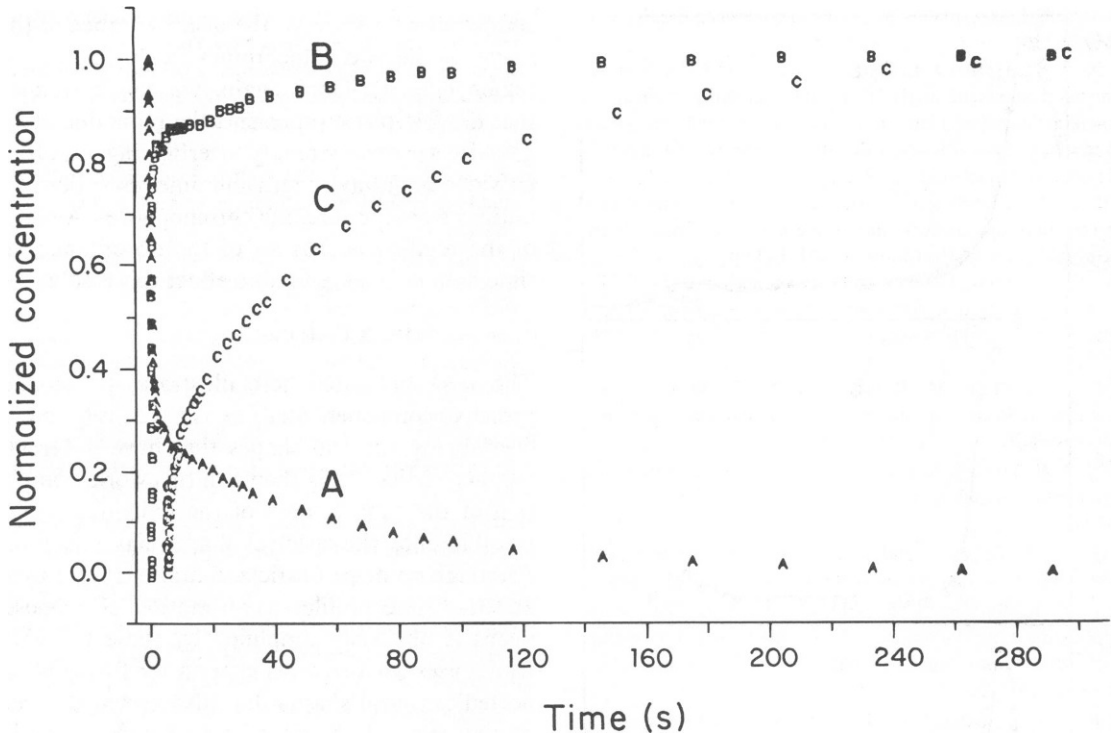

FIGURE 7 Concentration-time profiles for the reduction of the 424-nm species of resting cytochrome *c* oxidase by MPH. (*A*) Time course for the combined absorbance decay of oxidized aa_3; (*B*) growth of reduced cytochrome *a*; (*C*) growth of reduced cytochrome a_3.

concentration-time profiles: cytochrome a_3^{3+} reduction lags that of cytochrome a^{3+}. This, of course, explains the multiphasic kinetics of the increase in absorbance at 444 nm upon reduction because growth at this wavelength results from contributions from both cytochromes. Data obtained in the 400–650-nm wavelength region (Fig. 5) show via PCA that the reduced cytochrome *a* spectrum has a shoulder in the Soret region and also a higher contribution to the α band than does reduced cytochrome a_3. The analysis in this wavelength region also showed that in the 424-nm species of the resting enzyme, the spectral shapes of the oxidized cytochromes *a* and a_3 are similar, and thus become difficult to separate. A more detailed discussion of the quantitative aspects of the reduction kinetics of the two different forms of the resting enzyme we have used is presented elsewhere (Halaka et al., 1984).

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REFERENCES

Anderson, T. W. 1963. Asymptotic theory for principal component analysis. *Ann. Math. Stat.* 34:122–148.

Babcock, G. T., L. E. Vickery, and G. Palmer. 1978. The electronic state of heme in cytochrome oxidase II. *J. Biol. Chem.* 253:2400–2411.

Babcock, G. T., P. M. Callahan, M. R. Ondrias, and I. Salmeen. 1981. Coordination geometries and vibrational properties of cytochromes *a* and a_3 in cytochrome oxidase from Soret excitation Raman spectroscopy. *Biochemistry* 20:959–966.

Bellman, R. 1970. Introduction to Matrix Analysis. McGraw-Hill, Inc., New York. Second ed. 328 pp.

Blair, D. F., D. F. Bocian, G. T. Babcock, and S. I. Chan. 1982. Evidence for Modulation of the Heme Absorptions of Cytochrome *c* Oxidase by Metal-Metal Interactions. *Biochemistry.* 21:6928–6935.

Blumer, J. T., and H. F. Shurvell. 1973. Factor analysis as a complement to band resolution techniques. I. The method and its application to self-association of acetic acid. *J. Phys. Chem.* 77:256–262.

Brudvig, G. W., T. H. Stevens, R. H. Morse, and S. I. Chan. 1981. Conformations of oxidized cytochrome *c* oxidase. *Biochemistry.* 20:3912–2921.

Carithers, R. P., and G. Palmer. 1981. Characterization of the potentiometric behavior of soluble cytochrome oxidase by magnetic circular dichroism. *J. Biol. Chem.* 256:7967–7976.

Carter, K. R., T. M. Antalis, G. Palmer, N. S. Ferris, and W. H. Woodruff. 1981. Spectroscopic characterization of compound C and related derivatives of cytochrome oxidase. *Proc. Natl. Acad. Sci. USA.* 78:1652–1655.

Caughey, W. S., W. J. Wallace, J. A. Volpe, and S. Yoshikawa. 1976. Cytochrome *c* Oxidase. *In* The Enzymes, Vol. 13. P. D. Boyer, editor. Academic Press, Inc., New York. 299–344.

Cochran, R. 1977. Principal component analysis of rapid scanning spectroscopy experiments in chemical kinetics. Ph.D. thesis, Michigan State University, East Lansing, MI. 380 pp.

Cochran, R. N., and F. H. Horne. 1977. Statistically weighted principal component analysis of rapid scanning wavelength kinetics experiments. *Anal. Chem.* 49:846–853.

Cochran, R. N., and F. H. Horne. 1980. Strategy for resolving rapid

scanning wavelength experiments by principal component analysis. *J. Phys. Chem.* 84:2561–2567.

Cochran, R. N., F. H. Horne, J. L. Dye, J. Ceraso, and C. H. Suelter. 1980. Principal component analysis of rapid scanning wavelength stopped flow kinetics experiments on the liver alcohol dehydrogenase catalyzed reduction of *p*-nitroso-*N*,*N*-dimethylaniline by 1,4-dihydronieotinamide adenine dinucleotide. *J. Phys. Chem.* 84:1567–1575.

Coolen, R. B. 1975. A computer interfaced rapid scan stopped-flow system for the study of transients in enzyme reactions. Ph.D. thesis, Michigan State University, East Lansing, MI. 144 pp.

Coolen, R. B., N. Papadakis, J. Avery, C. G. Enke, and J. L. Dye. 1975. Computer-interactive system for stopped-flow kinetics with rapid scanning molecular absorption spectrometry. *Anal. Chem.* 47:1649–1655.

Greenwood, C., T. Brittain, M. Brunori, and M. T. Wilson. 1977. Chromous ion reduction of mammalian cytochrome oxidase and some of its derivatives. *Biochem. J.* 165:413–416.

Halaka, F. G., G. T. Babcock, and J. L. Dye. 1981. Kinetic distinction between cytochromes *a* and a_3 in cytochrome *c* oxidase. *J. Biol. Chem.* 256:1084–1087.

Halaka, F. G., G. T. Babcock, and J. L. Dye. 1982. Properties of 5-methylphenazinium methyl sulfate. *J. Biol. Chem.* 257:1458–1461.

Halaka, F. G., Z. K. Barnes, G. T. Babcock, and J. L. Dye. 1984. Reduction of cytochrome oxidase by 5,10-dihydro-5-methylphenazine: Kinetic parameters from rapid-scanning stopped-flow experiments. *Biochemistry.* 23:2005–2010.

Hartzell, C. R., and H. Beinert. 1974. Components of cytochrome *c* oxidase detectable by EPR spectroscopy. *Biochim. Biophys. Acta.* 368:318–338.

Hartzell, C. R., R. E. Hansen, and H. Beinert. 1973. Electron carriers of cytochrome oxidase detectable by electron paramagnetic resonance and their relationship to those traditionally recognized in this enzyme. *Proc. Natl. Acad. Sci. USA.* 70:2477–3481.

Hofrichter, J., J. H. Sommer, E. R. Henry, and W. A. Eaton. 1983. Nanosecond absorption spectroscopy of hemoglobin: Elementary processes in kinetic cooperativity. *Proc. Natl. Acad. Sci. USA.* 80:2235–2239.

Horie, S., and M. Morrison. 1963. Cytochrome *c* oxidase components. *J. Biol. Chem.* 238:2859–2865.

Lemberg, M. R. 1969. Cytochrome oxidase. *Physiol. Rev.* 49:48–121.

Malinowski, E. R., and D. G. Howery. 1980. Factor Analysis in Chemistry. John Wiley & Sons, Inc., New York. 251 pp.

Malmstrom, B. G. 1973. Cytochrome *c* oxidase: Some current biochemical and biophysical problems. *Q. Rev. Biophys.* 6:389–431.

Malmstrom, B. G. 1979. Cytochrome *c* oxidase structure and catalytic activity. *Biochim. Biophys. Acta.* 549:281–303.

Morse, R. H., and S. I. Chan. 1980. Electron paramagnetic resonance studies of nitrosyl ferrous heme complexes. *J. Biol. Chem.* 255:7876–7882.

Nicholls, P., and B. Chance. 1974. Cytochrome *c* oxidase. *In* Molecular Mechanisms of Oxygen Activation. O. Hayaishi, editor. Academic Press, Inc., New York. 479–534.

Papadakis, N., R. B. Coolen, and J. L. Dye. 1975. Variable temperature computerized dual-beam, rapid scanning stopped-flow apparatus for the study of air-sensitive systems and transients in enzyme reactions. *Anal. Chem.* 47:1644–1649.

Ritter, G. L., S. R. Lowry, T. L. Isenhour, and C. L. Wilkins. 1976. Factor analysis of the mass spectra of mixtures. *Anal. Chem.* 48:591–595.

Scott, R. A., and H. B. Gray. 1980. Cytochrome aa_3 electron-transfer reactions. Kinetics of hexaammineruthenium(II) reduction of the beef heart enzyme. *J. Am. Chem. Soc.* 102:3219–3224.

Shrager, R. I., and R. W. Hendler. 1982. Titration of individual components in a mixture with resolution of difference spectra, pKs, and redox transitions. *Anal. Chem.* 54:1147–1152.

Sylvestre, E. A., W. H. Lawton, and M. S. Maggio. 1974. Curve resolution using a postulated chemical reaction. *Technometrix.* 16:353–361.

Vadasdi, K. 1974. On determining the composition of species present in a system from potentiometric data. *J. Phys. Chem.* 78:816–820.

Vanneste, W. H. 1966. The stoichiometry and absorption spectra of components *a* and a_3 in cytochrome *c* oxidase. *Biochemistry.* 5:838–848.

Wikstrom, M., K. Krab, and M. Saraste. 1981. Cytochrome Oxidase, A Synthesis. Academic Press, Inc., New York. 198 pp.

Wikstrom, M. K. F., H. J. Harmon, W. J. Inglewood, and B. Chance. 1976. A Re-evaluation of the spectral potentiometric and energy-linked properties of cytochrome *c* oxidase in mitochondria. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 65:259–276.

Yonetani, T. J. 1960. Studies on cytochrome oxidase. *J. Biol. Chem.* 235:845–852.