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Spectral Relaxation of "Pulsed" Cytochrome c Oxidase

M. K. Abubakar*, M. Lawal and L. S. Bilbis

Department of Biochemistry, Usmanu Danfodiyo University, Sokoto, Nigeria.

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ABSTRACT: Cytochrome c oxidase was isolated from bovine heart using the method of Yonetani (1961). The enzyme was transformed into the more active "pulsed" form by complete reduction followed by subsequent reoxidation as described by Orii and King (1976). The spectral relaxation of the metastable "pulsed" conformation was monitored between 400 and 500nm. The time resolved spectra were analyzed using Singular Value Decomposition (SVD) as described by Henry and Hofrichter (1992). The results obtained indicate that the "resting" to "pulsed" conformational transition is global, involving the contemporaneous decay of the two chromophores, cytochromes a and a_3, within the enzyme and are discussed in terms of the putative bridging ligand between cytochrome a_3 and CuB.

INTRODUCTION

Cytochrome c oxidase (E.C. 1.9.3.1) is a membrane bound metalloenzyme which catalyzes the distal reaction of the respiratory chain, involving the four electron four proton reducation of dioxygen to water (Brunori *et al.*, 1988). In addition, in the mitochondria or in the enzyme reconstituted into liposomes, the reaction is accompanied by electrogenic separation of proton which is a result of both the proton pumping action of the enzyme and the scalar dioxygen reduction on the inner face of the inner mitochondrial membrane (Capaldi *et al.*, 1983). The enzyme is made up of two chemically identical haem, haem a, which acquire distinct chemical and spectroscopic properties by virtue of the different environments they occupy in the subunit 1 of the macromolecular complex (Brunori *et al.*, 1987). The low potential cytochrome a is low spin ($S = ½$) and bis-ligated to histidines 102 and 421 in the two axial positions (Hostler *et al.*, 1993). Cytochrome a_3 which is generally believed to be the site of the chemistry of dioxygen reduction and proton pumping (Babcock and Wikstrom, 1992), is high spin and pentacoordinate with histidine 419 as the proximal ligand (Hostler *et al.*, 1993).

The two metal centres are each associated with a Cu atom, CuA and CuB associated with cytochromes a and a_3 respectively. There is, however, evidence to believe that the CuA centre comprises two Cu atoms in the mixed valence state (Moore *et al.*, 1993). EPR and MCD studies have suggested that cytochrome a_3 and CuB form an antiferromagnetically coupled (S=2) binuclear centres (BNC) (Cheesman *et al.*, 1993). The nature of the ligand which mediates this overlap exchange interaction is, however, not known for certain.

The BNC has been reported to exhibit a considerable degree of spectral and functional heterogeneity, induced by disparately varied conditions such as pH, addition of formate or chloride and redox "recycling" (Moody *et al.*, 1991). However, the enzyme isolated from bovine heart has been reported to exist in two distinct conformation of the BNC which catalyze the oxidation of ferrocytochrome c with the same stoichiometry but which differ in the rate of intramolecular electron transfer (Antonini *et al.*, 1977). These two conformations are referred to as the "resting" and "pulsed" enzymes (Wilson *et al.*, 1977). The former term is used to refer to the oxidized enzyme as isolated, characterized by relatively slow rate of intramolecular electron transfer, while the latter term refers to the enzyme after full reduction followed by

*To whom correspondence should be addressed.

subsequent reoxidation. The two forms differ spectrally and in ligand binding kinetics (Morgan *et al.*, 1985).

Although the pulsed enzyme is known to be generated during turnover, the nature of this transition and its physiological significance are not fully understood.

This paper re-examined the kinetics of spectral relaxation of the pulsed enzyme by employing Singular Value Decomposition (SVD) (Hendler and Westerhoff, 1992) to analyze the generated time resolved absorption spectra for the transition. This is with the view to gaining some insight into the changes taking place in the BNC during the transition of the enzyme into the pulsed form. Previous report have suggested that the relaxation of the pulsed structure conforms to a simple two step sequential process of the type A>B>C (Orii and King, 1976).

MATERIALS AND METHODS

Bovine heart cytochrome c oxidase was prepared according to the method of Yonetani (1961). "Column prepared" pulsed enzyme was prepared as described by Orii and King (1976). The enzyme (230 µM, total haem) in 0.2 M phosphate buffer, pH 7.4, containing 2% sodium cholate was subjected to ten cycles of alternating vacuum and nitrogen gas and sealed under a slightly positive nitrogen pressure. The enzyme was reduced by the addition of 100 µl of 20 mM sodium dithionite. The mixture was allowed to stand on the bench at ambient temperature of 20°C for 20 minutes. The resulting reduced enzyme was applied on a G-25 column (1.5 x 9.0cm) pre-equilibrated with 10 mM Hepes-KOH buffer, pH 7.5, containing 0.1% tween-80 and was eluted with the same buffer to remove excess dithionite. The conversion of the enzyme to the pulsed form was confirmed by the position of its Soret peak centred around 430nm while its concentration was determined from its absorbance at 429nm using extinction coefficient of 78.6 μm^{-1} in line with the report of Brunori *et al.* (1983).

The solution of the resulting enzyme (16.5 µM, total haem) in the eluting buffer, was taken into a 1 cm cuvette and a set of time resolved spectra were recorded at intervals of 15 minutes between 400 and 500nm.

The generated absorbance-wavelength-time data matrix was analyzed using 386-MATLAB (The Mathworks, Inc., Natick, MA) software, while the time courses were fitted to specific models using the Matlab functional minimization package as described by Antonini *et al*, (1993).

ANALYSIS OF RESULTS

SVD is essentially a matrix transformation procedure which ultimately enables the representation of one matric by another of lower rank. An indepth theoretical description of this procedure has been provided by Henry and Hofrichter (1992).

Briefly, for an m x n matrix, A, there is exist two factor matrices $\underline{U}$ and $\underline{V}$ such that:

$$\underline{A} = \underline{U}\,\underline{S}\,\underline{V}^T \text{ --------------(1)}$$

$\underline{U}$ is an m x n matrix having the property of $\underline{U}^T\underline{U} = I_n$ where I_n is an m x m identify matric and $\underline{V}$ is an m x n matrix such that $\underline{V}^T\underline{V} = I_n$. S is a diagonal matrix of decreasing non-negative elements called the singular values of A.

It can be shown that:

$$\underline{A}^T\underline{A} = (U\underline{SV}^T)^T\ \underline{USV}^T$$
$$= \underline{VSU^TUSV^T}$$
$$= \underline{VS^2V^T}$$

Consequently, the squares of the diagonal elements of S are the eigenvalues of $\underline{A}^T\underline{A}$ with the column $\underline{V}$ as the eigenvectors. Hendler and Schrager (1993) have demonstrated that the matrix $\underline{V}$ preserves the row space information in $\underline{A}$. The column $\underline{U}$ may similarly be shown to be the eigen values of $\underline{AA}^T$ and, therefore, preserve the column space information in $\underline{A}$.

Suppose a set of 20 time-resolved spectra are collected at 100 wavelengths. The data may be represented as a matrix $\underline{A}$ with 100 rows and 20 columns (i.e. 100 x 20). The columns of $\underline{A}$ contain only spectral information, while the rows contain information on the time courses at a single wavelength. The matrix may then be analyzed using SVD. The column of $\underline{U}$ contain only spectral information and are called the eigenspectra of $\underline{A}$, while the columns of $\underline{V}$ contain only information on the time courses of the corresponding $\underline{U}$ columns. The singular values, on the other hand, are the relative weights with which the individual $\underline{U}$ columns contribute to the observed spectral change. These values, coupled with autocorrelation analysis, may be used to determine the effective rank of the data matrix, i.e. the number of linearly independent eigen-spectra necessary to describe the data set. The value of autocorrelation coefficient less than 0.5 for any component is generally considered to indicate the ppreponderence of noise over signal and such columns may be discarded. Once the number of specially distinct species involved in the process has been decided, the true spectra of the species with any significant contribution to the spectral behaviour

in A, may be recreated from another matrix whose elements are obtained from the amplitude of the global fitting of the V columns to a specific mechanism (see Hendler and Schrager, 1993).

RESULTS

The time resolved difference spectra (pulsed minus resting) for the spectral relaxation of the pulsed enzyme are shown in Figure 1. The difference spectra were chracterized by crests around 430nm and troughs at 412nm. The output of the SVD analysis are shown in Figure 2, 3 and 4. Three spectrally significant components were discerned from the S-values of U and V vectors and their autocorrelation functions (Table 1).

These values indicate that at least four spectrally distinct species (3 from SVD plus 1 for the reference state) are involved in the relaxation process. The first three V - columns were fitted to a sequential mechanism of form A>B>C>D (Figure 3) (cf. Orii and King, 1976) and to a parallel mechanism of the form A>B and C>D (Figure 4). The first order of rate constants obtained from the fitting to sequential and parallel mechanism were 4.33×10^{-4}, 1.17×10^{-4} S^{-1} and 6.30×10^{-4} S^{-1}, and 1.32×10^{-4} and 1.33 x 10-5 S^{-1} respectively. A better conformity to the experimental data was obtained, however, with the parallel mechanism as judged from the amlitude of the input data matrix in relation to that in the recreated spectra (Figure 4). The recreated difference spectra had peaks centred at 430nm and 438 nm with trough at 412nm. It should be noted, however, that the parallel mechanism is consistent with the spectral decays of two chromophores which absorb in the Soret region, cytochromes a and a3.

In order to ascertain the validity of the parallel mechanism, it was deemed necessary to test certain predictions of the SVD output (Figure 4) using the original data set as the canon. These predictions are:

1. The difference spectrum of cytochrome a_3 (the spectrum on the blue end in Figure 4, the rationale for this assignment has already been explained elsewhere (Abubakar, 1994)< exhibits an isosbestic point at 447 nm.

2. At this isosbestic point (447nm), the difference spectrum due to cytochrome a is expected to decay exponetially with a first order rate constant comparable to one of the values obtained from the parallel fitting. The difference spectra obtained using the sequential mechanism, on the other hand, predicts that the time course at 447nm, in the original data matrix, be a summed contribution of the spectral decays of the species A, B and C (Figure 3).

Figure 5 illustrates the plot of the absorbance change at 447nm in the original data set. Insert to the figure is the semilog plot of the time resolved absorbance. The plot exhibited an exponential decay with an estimated first order rate constant of 3.74 x 104 S^{-1}. This value is in good agreement with the value of 4.33×10^{-4} S^{-1} determied from the global fitting of the three V-columns. This was considered as another important proof of the validity of the parallel mechanism.

DISCUSSION

The transformation of cytochrome c oxidase from the resting to the pulsed form has been shown to be characterized by a bathochromically shifted Soret band centred around 430nm (cf. 418nm for the resting enzyme). The electronic origin of the stabilization of the optical transition dipole moment for this in-plane polarized > * transition in the porphyrin ring (Lynch *et al.*, 1991) is, however, a matter of conjecture. Also less understood is the origin of the enhanced rate of intramolecular electron transfer within the enzyme (Brunori *et al.*, 1994). However, the optical transition in cytochrome a_3 consequent upon this redox "recycling" of the enzyme is similar to that observed in the high to low spin transition induced by the binding of CN to oxidized cytochrome a_3 (Wilson *et al.*, 1994). This binding, among other things, results in the bathochromic shift of the Soret peak of the same extent to that observed when the enzyme is pulsed. It may,therefore, be conjectured that cytochrome a_3 in the pulsed enzyme is low spin.

It must be noted, however, that in general, low spin transition in haem proteins has an important redox consequence; electron transfer to low spin haem proteins requires less electronic reorganisation (Cheesman *et al.*, 1991). Coupled with this is that the enhanced crystal field splitting energy in low spin ferrhaem complexes places the energy of iron t2g orbitals at or near parity with that of the porphyrin * orbitals located at the edge of the ring (Moore and Pettigrew, 1990). The mixing of these orbitals results in the existence of electron cloud between the central iron and the edge of the porphyrin ring (Marcus and Sutin, 1985) which enhances the possibility of facile electron transfer to and from the iron centre via the haem edge. All the aforementioned tend to increase the rate of electron transfer to and from such centres in accordance with the dictates of Marcus theory (Moser *et al.*, 1992).

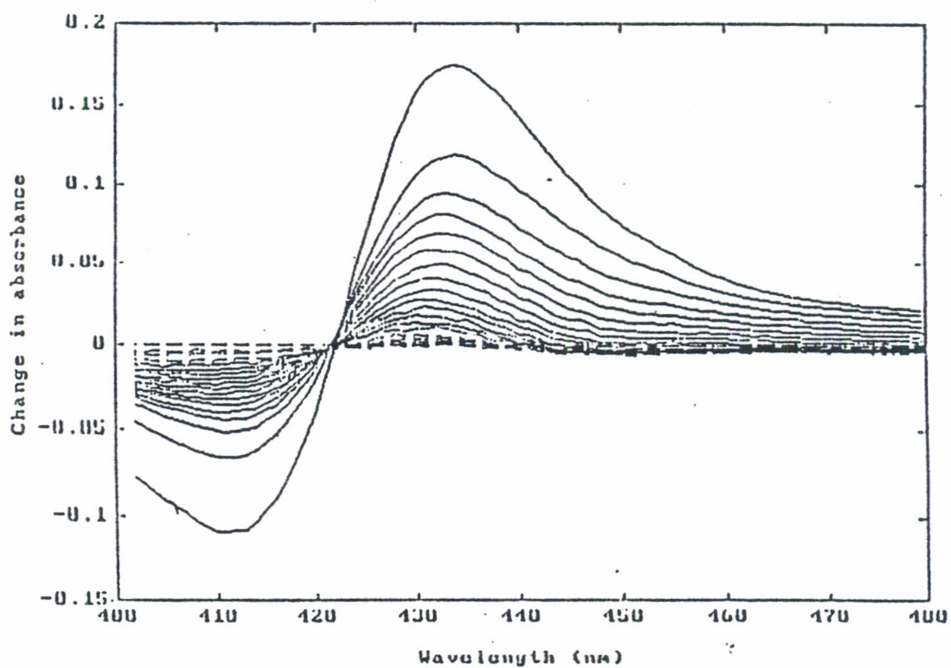

Figure 1: Time resolved difference spectra ["pulsed minus resting for the relaxation of pulsed" cytochrome c oxidase (16.5 um total haem)] in 0.2M phosphate buffer, pH 7.5. Successive spectra were measured at intervals of 15 minutes at 20°C.

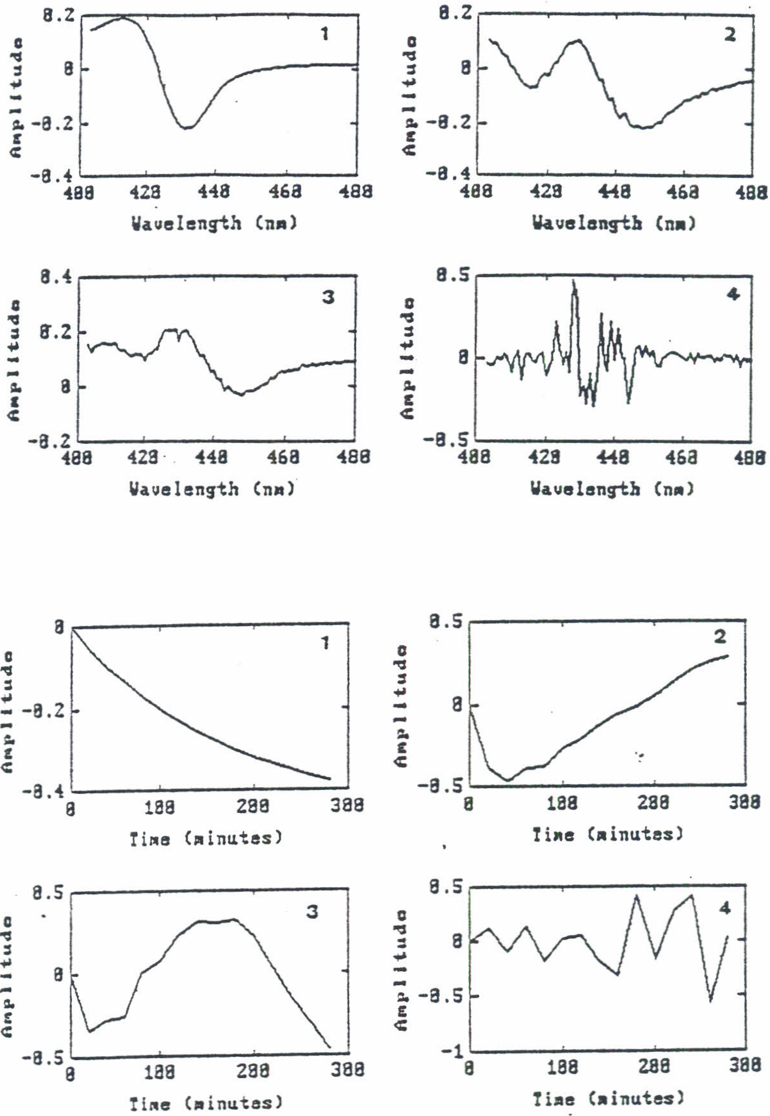

Figure 2: An illustration of the first four columns of U (top panel) and V (bottom panel) obtained from SVD analysis of the time-resolved difference spectra shown in Figure 1.

Table 1: A summary of the singular values and autocorrelation functions obtained from the SVD analysis of the difference spectra for the relaxation of pulsed cytochrome c oxidase.

Column	S-Value	Autocorrelation (V)	Function (U)
1	4.5525	0.9924	0.9589
2	0.2007	0.9861	0.7633
3	0.0953	0.9737	0.5547
4	0.0161	0.9396	0.4420

The resting to pulsed conformational transition and the associated changes in spectroscopic and redox properties have long been documented (Brunori *et al.*, 1983). The observed stabilization of the optical transition dipole moment, of the order of 6.8KJ/mole (416 to 418nm), for cytochrome a3, and the enhanced rate of intramolecular electron transfer within the enzyme may all be the result of some electronic reorganization in the chromophore the nature of which is poorly understood (Wilson *et al.*, 1981). We wish to posit, in this communication, that the optical and electron transfer properties of the pulsed enzyme may be rationalized if the following observations and assumptions are taken into cognizance:

1. The antiferromagnetic coupling between cytochrome a_3 and CuB is mediated by a strong field ligand weakly bound to cytochrome a_3 and CuB. Electron paramagnetic resonance and kinetic studies have suggested this ligand could be histidine (See Brunori *et al.*, 1994 and reference contained therein).

2. CuB is the entry port to the first electron into the BNC (Wilson *et al.*, 1994).

3. Reorganization of the BNC entails the displacement of the bridging ligand and its subsequent binding to cytochrome a_3 to induce a low spin 430nm optical transition (Brunori *et al.*, 1994).

4. The essentially overlap super-exchange (antiferromagnetic) interaction between the two metal centres increases the bond length between the central iron atom in cytochrome a_3 and the bridging ligand and hence decreases the low spin character of the haem.

Figure 1 illustrates the spectral decay of the pulsed enzyme into the resting form in agreement with the results of other workers (Orii and King, 1976). The thermodynamic implication of this is that the "pulsed" structure is metastable and relaxes back into the resting form at a rate determined by kinetic factors. We wish to posit that if the transient binding of the otherwise bridging (strong) ligand to cytochrome a_3 subsequent upon "pulsing" the enzyme is granted, then the relaxation of the pulsed conformation would entail the re-establishment of the bridge to CuB which is attended by a decrease in the low spin character of the haem. The observed enhancement of the rate of intramolecular electron transfer within the enzyme may, therefore, be attributable to the reorganization of CuB and the low spin character of cytochrome a_3 due to the reasons outlined above.

The observed spectral decay attributable to cytochrome a in the deconvoluted spectra for the relaxation of the pulsed enzyme (Figure 4) is also another intriguing observation. This is because although the reduction and subsequent reoxidation of cytochrome a_3 is generally believed to be the trigger of resting to pulsed transition, this observation points to the global nature of this transition. The implication of this is that conformational changes in cytochrome a_3 are transmitted to cytochrome a via the mechanical connection of the intervening peptide. This is easily rationalized in the light of the above if it is realized that the oscillations in the spin state of cytochrome a3, its attendant expansion and shrinkage of the elctron cloud around the iron, and the reversible displacement of iron out of the plane of the haem engenders a change in the coordination geometry around cytochrome a3. The trans effect exerted by such changes on histidine 421 may, therefore, be transmitted, allosterically, to histidine 419 via the half turn of helix which connects the two haems (Hosler *et al.*, 1993) and thereby modifying the environment and hence the spectrum of cytochrome a.

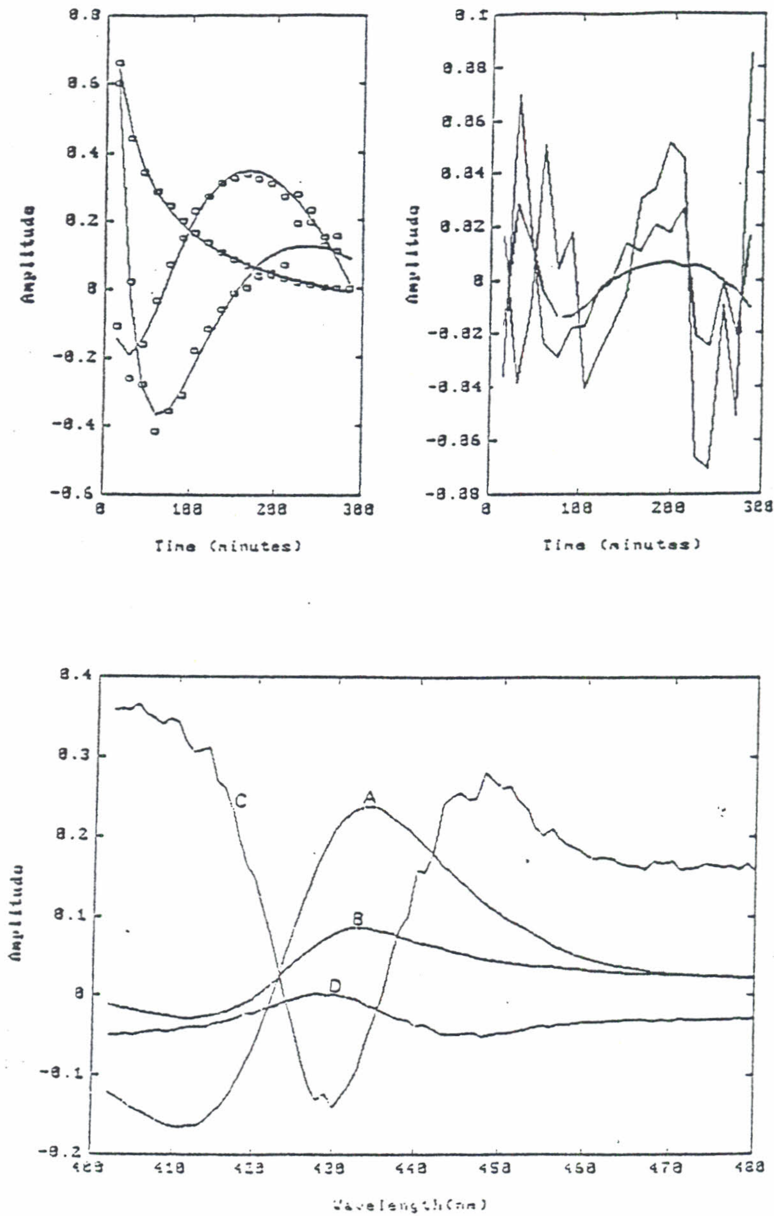

Figure 3: Top panel: The results of the fits (left) and residual (right) to a sequential mechanism involving four species of the first three V-volumn (Figure 2), for the time-resolved difference spectra in Figure 1.

Bottom panel: The difference recreated from the first three U-column, their corresponding singular values and the amplitudes obtained from the fits.

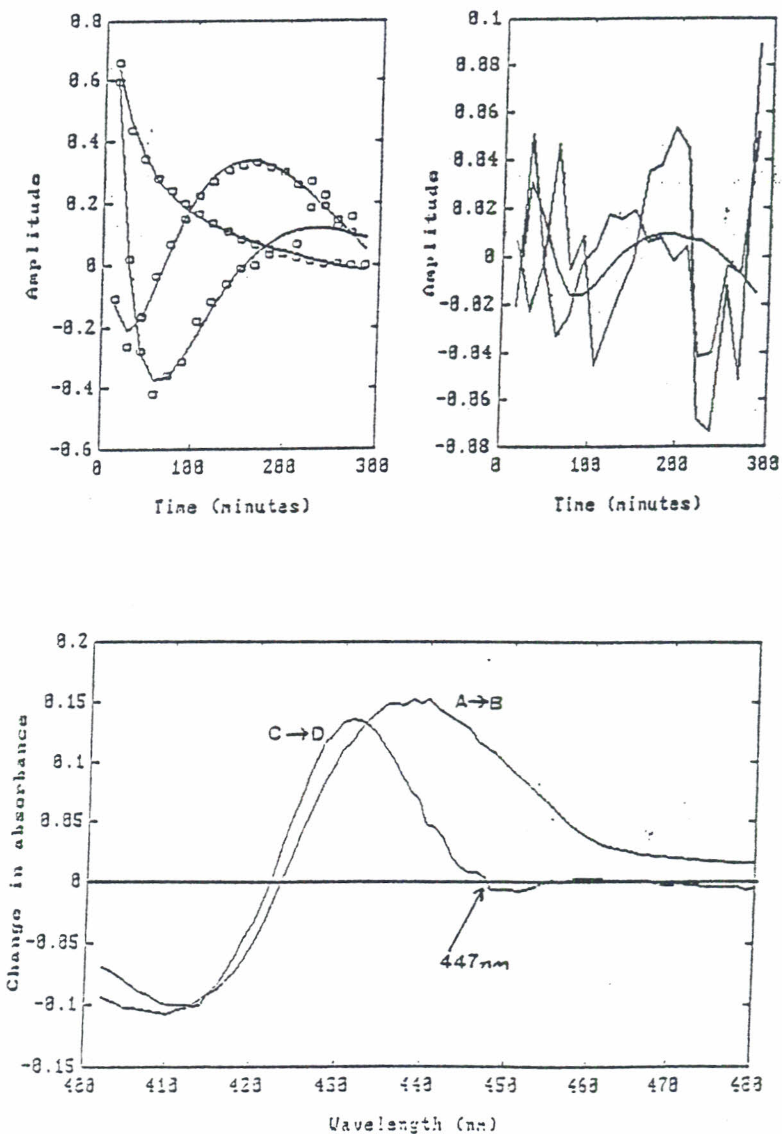

Figure 4: Top panel: The results of the fits (left) and residual (right) to a parallel mechanism involving four species of the first three V-column (Figure 3), for the time-resolved difference spectra in Figure 1.

Bottom panel: The difference recreated from the first U-column, their corresponding singular values and the amplitudes.

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