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Potentiometric and optical resolution of cytochromes *c* and c_1 in purified mitochondria from higher plant tissue, *Solanum tuberosum*

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Abstract. Mitochondrial cytochromes *c* and c_1 have long been considered indistinguishable from a potentiometric point of view. By combining rapid scan spectrometry to run redox titrations with numerical analysis using a generalized Nernst equation, it was possible to resolve cytochrome *c* and c_1 midpoint potentials in yeast and mushroom mitochondria. In the reported work, this approach has been applied to purified mitochondria from higher plant tissue (*Solanum tuberosum* L.). The rapid scan spectrometric technique provided clear evidence of reversible base line changes monitored by redox potential changes. The basic mechanism responsible for this modification in the mitochondria optical properties remains to be defined. However, we suggest that this phenomenon could play a regulatory role in the overall electron transfer process. It is necessary to make an initial correction of the recorded spectra prior to numerical analysis. When this is done, two midpoint potential values are resolved by running analyses in the 550–555 nm range: 283 ± 3 mV and 213 ± 11 mV. They are identical to the ones found for cytochromes *c* and c_1 in yeast and mushroom mitochondria. The individual difference α bands were resolved by running analyses at each wavelength of the corrected spectra, the resolved midpoint potentials being kept fixed. This approach, the only one to date which has succeeded in resolving mitochondrial cytochrome *c* and c_1 midpoint potentials, is discussed with respect to other methods. Limitations are pointed out.

Key words: Potentiometry, cytochrome, plant mitochondria, rapid scan spectrometry

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Abbreviation: Mops: morpholinopropane sulfonate

Introduction

The electron flux which proceeds in the mitochondrial respiratory chain from NADH-dehydrogenase up to oxygen releases free energy which is partially converted in building up a proton electrochemical potential in the intermembrane space at the expense of the matrix space. This electron flow occurs through a sequence of events, not in a single step. Redox carriers involved in each step can be of very different nature (flavoprotein, iron sulfur protein, ubiquinone, heme-protein, copper atom) and very different in topology as well, for instance within the membrane or in the aqueous phase (Lehninger 1982).

Complete understanding of the overall process implies the knowledge of the standard redox potential E° (or at least the midpoint potential E_m) of the involved components in their mitochondrial environment.

This objective has generated a large number of investigations based on a procedure extensively developed by Dutton and Wilson (1970, 1974). As to the *c*-type cytochromes, overlapping of their optical and potentiometric properties, when bound to the mitochondrial membrane, long prevented resolution of their in situ midpoint potentials. They were therefore considered as indistinguishable (Dutton et al. 1970; Dutton and Storey 1971).

The derivation of a generalized Nernst equation (Denis et al. 1973) together with spectra recording by a suitable rapid scan spectrometric technique during redox titrations, opened they way to E_m resolution, by numerical analysis of titration curves, for cytochromes *c* and c_1 in yeast mitochondria (Denis et al. 1980b).

In the present paper we report the resolution of cytochrome *c* and c_1 midpoint potentials in purified mitochondria from higher plant tissue, *Solanum tuberosum* L., together with their related spectral resolution in the α range.

Other methods developed for resolving potentiometric and optical properties of respiratory system

components are also discussed. Limitations of these methods and the present one as well are pointed out.

Materials and methods

Biochemical methods

Mitochondria were extracted from potato tubers (*Solanum tuberosum* L.) and purified by centrifugation on a discontinuous density sucrose gradient as described elsewhere (Richaud and Denis 1984). The average protein content of the purified preparation, almost completely deprived of microsomal membrane contamination, was about 80 mg · ml^{-1}. The mitochondria integrity was controlled by the combined measurements of oxygen uptake and pH changes during substrate oxidation as reported by Ducet (1980). Purified mitochondria were suspended in a medium comprising 0.18 *M* mannitol and 60 m*M* potassium phosphate buffer, pH 7.2.

Rapid scan spectrometry

Absorbance changes during the redox titrations were collected with the home built rapid scan spectrometer CD66 (Denis 1976; Denis and Ducet 1975) used in the dual beam mode. The scanning speed was about 80 spectra per second. Spectra were recorded after equilibration of the redox potential and were stored on magnetic tape for further analysis. Each stored spectrum resulted from the accumulation of 400 successive spectra each defined by 128 data points from 585 to 520 nm. The digitization time for each data point was 7 μs. The signal to noise ratio was improved by this averaging procedure without increasing overall titration time. The instrumental base line was recorded initially and was automatically subtracted from each averaged spectrum prior to storage on tape. Base line changes occurring during the redox titrations were empirically corrected by calculating the absorbance values with respect to a straight line defined by data points at 575 and 540 nm (Denis et al. 1980b).

Potentiometric measurements

Potentiometric measurements were conducted according to the method of Dutton et al. (1970) and as defined previously (Denis 1976; Denis et al. 1980a). Redox potential values were collected through a combined platinum calomel electrode (PK 149, Radiometer, Cogenhagen, Denmark) connected to a millivoltmeter (mini 5000, Tacussel, Lyon, France). The electrode was fitted into a special absorbance cuvette (18 mm optical path). The suspensions were kept homogeneous by means of a glass stirrer. The main advantage of this device over magnetic stirrers is that it eliminates electromagnetic perturbations on the electrode as well as those on the photomultiplier tube. In all experiments, the sample temperature was kept at 20.00 ± 0.05 °C using a thermoregulated bath (model 850112, Braun, Melsungen, FRG). Strict anaerobiosis was maintained by a continuous stream of ultrapure argon purified by bubbling through a vanadyl sulfate column (Labeyrie 1963).

Samples to be titrated were made of mitochondria suspended in 0.1 *M* Mops buffer, pH 7.2 (Sigma, La Verpillière, France), containing 0.6 *M* sorbitol (Serlabo, Paris, France). The mitochondrial suspensions were supplemented with a series of redox mediators (Serlabo, Paris, France) the amounts of which are defined in figure legends: diaminodurol, $FeCl_3$-EDTA, pyocyanine, duroquinone and 2-hydroxy-1,4-naphtoquinone. The oxidant (0.1 *M* potassium ferricyanide) and the reductant (0.5 *M* dithionite) were prepared in deaerated 0.1 *M* Mops buffer and delivered with an airtight 500 μl micrometer syringe (Agla, London, UK).

Experimental precisions were in the range 0.5 to 1.0 $A \times 10^3$ and 0.5 to 1.0 mV for the combined absorbance and redox potential measurements, respectively.

Data analysis

Numerical analysis of the combined absorbance and redox potential measurements was based on a generalized Nernst equation (Denis et al. 1973). At redox equilibrium and for a given wavelength, absorbance A of the titrated suspension is directly expressed with respect to redox potential E_h by the following relationship (Denis 1976; Denis et al. 1980a):

$$A = A_r + \sum_{j=1}^{p} DA_j [1 + \exp((E_{mj} - E_h)/K_j)]^{-1} \qquad (1)$$

with $K_j = R\,T/n_j F$

where p is the assumed number of redox components contributing to the titration curve, E_{mj} is the midpoint potential of the jth component at the defined pH and temperature of the experiment; n_j is the number of electrons involved in the redox process of the jth species; T is the absolute temperature of the sample, $R = 8.314$ J K^{-1} and $F = 96{,}493$ C. A_r is the sample absorbance in the fully reduced state; A_r represents the sum of p terms: $l\,C_j\,\varepsilon_{rj}$ where l is the optical path length, C_j is the concentration of the jth component and ε_{rj} its molecular extinction coefficient for the observation wavelength when in reduced form.

$DA_j = l\,C_j\,\Delta\varepsilon_{j_{ox-red}}$ is the maximum absorbance change of the jth component at the considered wavelength; $\Delta\varepsilon_{j_{ox-red}}$ is the difference extinction coefficient of the jth component, in the sense oxidized minus reduced, at the observation wavelength. All redox potential values are referred to the normal hydrogen electrode. In the reported analyses, n_j was fixed at 1 electron and the parameters to be fitted were A_r, DA_j and E_{mj}.

The theoretical possibilities of resolving 2 close E_m values by using Eq. (1) have been detailed previously along with the computation methods (Denis 1976; Denis et al. 1980 a). Three statistical criteria of analysis have to be satisfied to ensure good agreement between experimental and theoretical values: (*i*) the overall standard deviation of the fitted function must be in the range of the experimental deviations; (*ii*) the parameters must be well defined and (*iii*) the deviations between experimental and calculated values must be randomly dispersed (Neau 1979; Neau and Peneloux 1981).

The treatment of the data stored on tape and their analysis were performed with a CII 10070 computer as reported previously (Denis et al. 1980 a). The experimental points and the fitted theoretical titration curves were displayed graphically by a Benson 122 plotter, as were the three dimensional displays of complete titration sets of spectra built with the CARTOLAB software.

Results

Redox titrations were carried out in both oxidative and reductive directions, in potential ranges encompassing complete redox changes for mitochondrial *c*-type cytochromes, i.e. 100–400 mV.

In all experiments, wavelength dependent changes in the opacity of the mitochondrial suspension occurred throughout the monitoring of the titrations as illustrated in Fig. 1 A. In this three dimensional plot, curves are difference spectra, all referred to the absorbance level at 358.1 mV where cytochromes *c* and c_1 are fully oxidized. Spectra are displayed at regular graphic intervals in the order of their acquisition. Therefore, the axis reporting redox potential values must not be considered as defining a linear scale. However, the redox potential steps being of comparable magnitude (5–8 mV), the three dimensional plot of Fig. 1 A provides a good visualization of optical changes during the course of a complete cycle in potential.

It is worth noting, in Fig. 1 A, that the first step of the reported titration is not located at one end of the scanned potential range. Such a constraint is released by the above handling of digitized spectra. As outlined

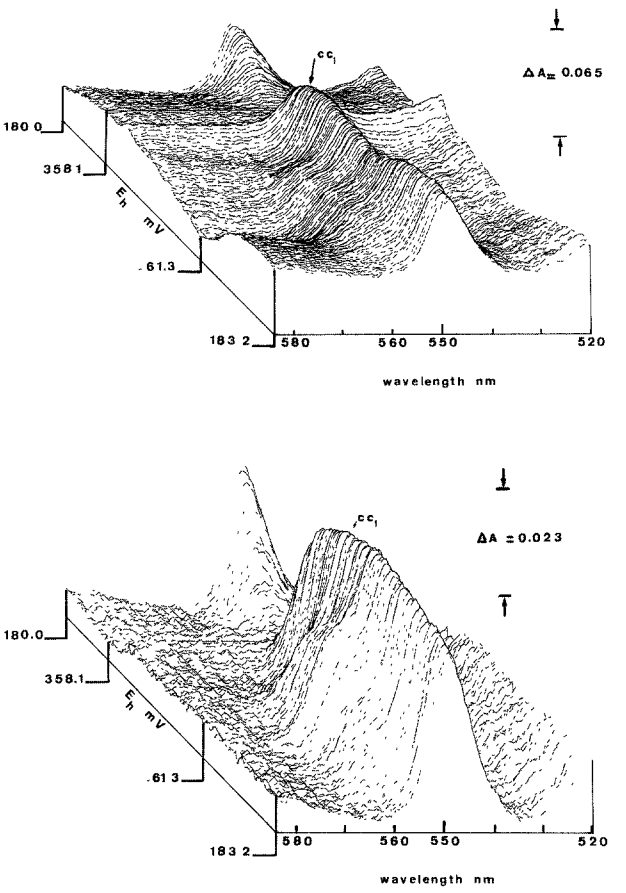

Fig. 1 A and B. Three dimensional display (absorbance, wavelength, redox potential) of a redox titration of mitochondria purified from higher plant tissue, *Solanum tuberosum* L. Scan speed, 80 spectra per s; digitization time, 7 µs per point, 128 points per spectrum. Each recorded spectrum resulting from the accumulation of 400 successive spectra, was automatically corrected for apparatus base line. Difference spectra were calculated with the spectrum recorded at 358.1 mV as reference. They are displayed in the order of their acquisition at regular graphic intervals. Therefore, the third dimension related to redox potential cannot be regarded as a linear redox potential scale. However, the regular graphic intervals and the choice of the potential steps help the three dimensional representation visualizing the behavior of *c*- and *b*-type cytochromes during the titration. In **A** spectra are not corrected for changes in the mitochondrial suspension base line; in **B** they are. Sample: 0.9 mg protein ml^{-1} purified mitochondria in 0.1 *M* Mops buffer, pH 7.2, 0.6 *M* sorbitol; uncoupler, 10 µ*M* carbonyl cyanide trifluoromethoxyphenylhydrazone; mediators, 0.477 m*M* diaminodurol, 40 µ*M* $FeCl_3$-EDTA, 5 µ*M* pyocyanine, 8 µ*M* duroquinone, 10 µ*M* 2-hydroxy-1,4-naphtoquinone; temperature, 20.00° C

previously (Denis et al. 1980 b), we have minimized the spectral distortion due to base line changes by estimating the cytochrome absorbance contributions with respect to a straight base line connecting data points at 575 and 540 nm. Figure 1 B represents the same set of spectra as in Fig. 1 A after such a correction. Numerical analysis was always done after correction of the recorded data set for base line changes. Titration

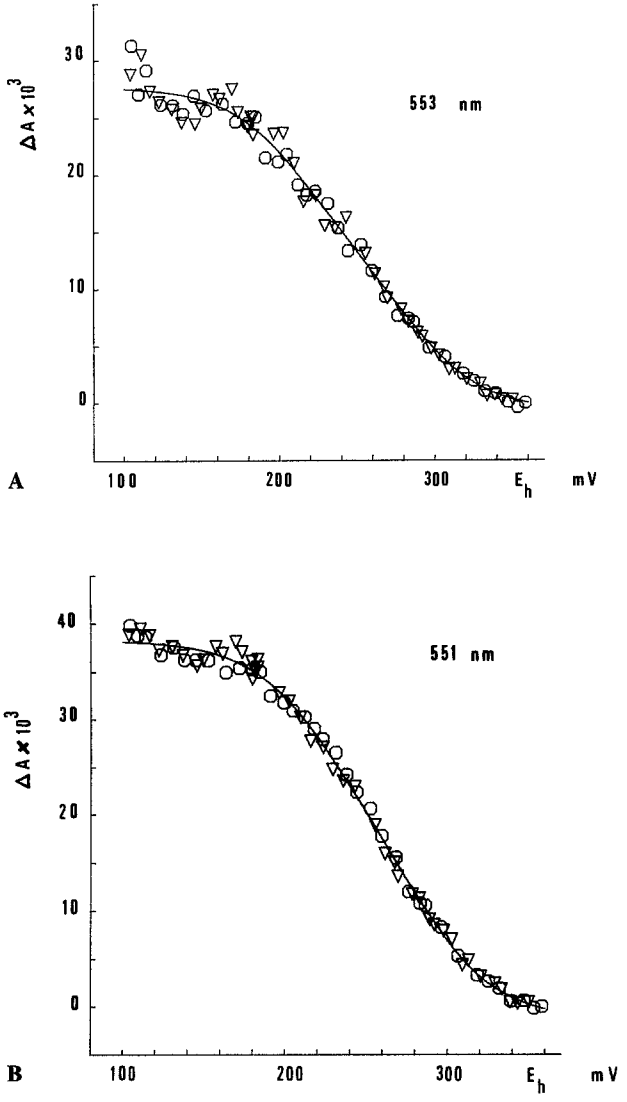

Fig. 2 A and B. Mitochondrial cytochrome c and c_1 midpoint potential resolution by numerical analysis of titration curves according to Eq. (1). **A** and **B** are typical computer print-outs of titration analyses in the 100–400 mV range, here at 553 and 551 nm respectively. Experimental points correspond to reductive (▽) and oxidative (○) titrations. Continuous lines are the best fits of the data obtained using the generalized Nernst relationship (Eq. (1)). Parameters determined in A are: $E_{m1}=213.76$ mV, $E_{m2}=284.22$ mV, $DA_1=-14.11\ A\cdot 10^3$, $DA_2=-14.48A\cdot 10^3$, $Ar=27.69\ A\cdot 10^3$; $n_1=n_2=1$ electron were fixed. In **B** the determined parameters are: $E_{m1}=223.81$ mV, $E_{m2}=284.65$ mV, $DA_1=-18.48\ A\cdot 10^3$, $DA_2=-21.07\ A\cdot 10^3$, $Ar=38.21\ A\cdot 10^3$; $n_1=n_2=1$ electron were also fixed. Experimental conditions as in Fig. 1

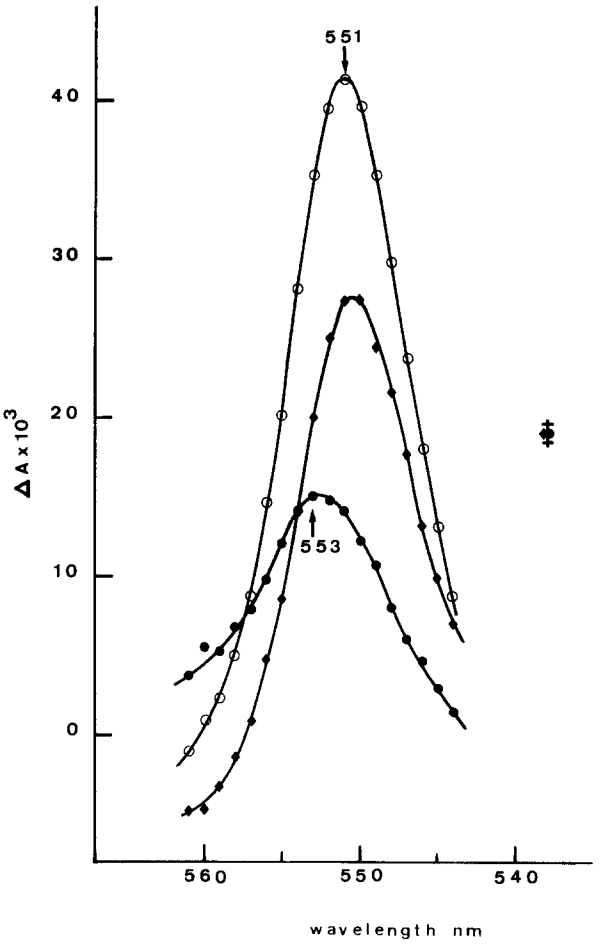

Fig. 3. Difference spectra resolution in the α range for mitochondrial cytochromes c and c_1 on the basis of their resolved potentiometric properties. The individual difference spectra (■——■) and (●——●) were calculated by processing the analysis at the successive wavelengths of spectra displayed in Fig. 1 B. The midpoint potential values were fixed to 285 and 215 mV respectively for cytochromes c and c_1. The sum of the resolved individual contributions is represented by open circles (○——○). Experimental conditions as in Fig. 1

curves at selected wavelengths were derived from data bases like the one in Fig. 1 B by collecting the related absorbance values.

Typical titration curves are shown in Fig. 2 A and B. Experimental points are represented by open circles for oxidation steps and open triangles for reduction ones. Note that the lower value of the analysed potential range was set at 100 mV where cytochromes c and c_1 are at least 99% reduced. Going further down in potential would have introduced optical interference from the b-type cytochromes whose increasing reduction is clearly visible in Fig. 1 A and B when redox potential is monitored down to −61.3 mV. The good superimposition of both titration protocols reflects a good reversibility of electron transfer across this segment of the respiratory chain under the above experimental conditions and indicates that good redox equilibrium is attained at each step.

Continuous lines correspond to the best fit of the data by the theoretical relationship $A(E_h)$, Eq. (1). Analysis of titration curves such as the ones in Fig. 2 A and B allowed the resolution of two species with $E_{m1}=213\pm 11$ mV and $E_{m2}=283\pm 3$ mV. The

ratio of their respective absorbance changes at 550 nm is 1/2. We must point out that the above deviations are simply indicative of the dispersion of the determined E_m values and do not characterize reproducibility which would have required a greater number of experiments.

The resolved E_m values were determined by running the analysis on titration curves belonging to the range 550–555 nm where overlapping of individual optical contributions is maximum. Indeed, the ratio of these contributions varies between 0.5 and 1 in that spectral range, which is in favor of a better E_m resolution than domains where one contribution would be 10% or less of the overall absorbance change (Denis et al. 1980 a). Then, both E_m parameters were fixed and the individual difference spectra in the α region were calculated by processing the analysis at each wavelength of the recorded spectra, the output of which is DA_1 and DA_2 at each wavelength (see Eq. (1)). The fixed E_m values from which the resolved α bands in Fig. 3 were derived, are 215 and 285 mV respectively for cytochromes c_1 and c.

The result of this spectral resolution applied to the data set of Fig. 1 B is presented in Fig. 3. The two resolved α bands (full circles and full squares) clearly identify the resolved species with cytochrome c_1 and cytochrome c respectively. The band with its peak position at 551 nm (open circles) is the sum of the resolved α bands and represents the maximum absorbance change of the mitochondrial suspension in the wavelength range analysed.

Discussion

Figure 1 A emphasizes previous reports of changes in optical properties during redox titrations of mitochondrial suspensions (Denis et al. 1980 b; Denis and Gallinet 1981) as well as bacterial respiratory systems (Hendler and Shrager 1979). Dealing with highly purified mitochondria provides a new insight on this phenomenon. Indeed, despite the fact that we still do not know the real mechanism by which physical features of the membraneous systems would be affected by the redox state of the electron transport chain, Fig. 1 A shows that this effect is a reversible one. This optical phenomenon probably expresses a regulatory process and, as such, would deserve further investigation.

The wavelength dependence of the base line changes occurring along with the redox titrations makes necessary a preliminary correction of the recorded spectra which cannot be satisfactorily achieved by dual wavelength spectrometry as outlined by Denis et al. (1980 b). In the absence of a theoretical basis for this correction, the empirical approach here applied seems quite appropriate when considering Fig. 1 B. In the analysis of titration curves like the ones of Fig. 2 A and B, we fixed to 1 electron the value of parameters n_j. Trying to fit n_j would be unjustified and misleading for the other parameters since n_j is well established for the mitochondrial c-type cytochromes.

The resolved E_m values for cytochromes c and c_1 in higher plant mitochondria, respectively 283 ± 3 mV and 213 ± 11 mV, are identical to the ones determined in yeast mitochondria 285 ± 5 mV and 220 ± 10 mV (Denis et al. 1980 b), and in mushroom mitochondria 292 ± 5 mV and 210 ± 10 mV (Denis and Gallinet 1981). This would reflect conservation of these potentiometric properties through all eukaryotic cells despite differences in amino acid sequences (Ducet 1985).

The resolution power of the present approach stems from (*i*) an appropriate model, Eq. (1), a generalized Nernst relationship involving direct measurements, A and E_h (Denis 1976; Denis et al. 1980 a), and not intermediate variables (Hendler et al. 1975). Recently, Eq. (1) has been adopted by other groups (Van Wielink et al. 1982; Shrager and Hendler 1986; Gradin and Colmsjo 1987); (*ii*) numerical analysis of the titration curves together with the use of statistical criteria as defined in Materials and methods; (*iii*) the use of rapid scan spectrometry which provides a fully coherent data base. Indeed, the short acquisition time (5 s) guarantees that absorbance values of a given spectrum can be referred to a common E_h value and that all titration curves derived from the same data base, such as the one in Fig. 1 B, are fully consistent since they are related to the same sample and are obtained from a single experiment.

The above three conditions were not available in the earlier work of Dutton and Storey (1971) which explains why these authors could not resolve the E_m values of cytochromes c and c_1 in higher plant mitochondria. Both cytochromes were also found to be indistinguishable in mitochondria from beef heart (Dutton et al. 1970) and from *Paramecium tetraurelia* (Doussière et al. 1979). To our knowledge, the present approach is the only one up to now which is able to resolve the midpoint potentials of the mitochondrial cytochromes c and c_1.

To resolve the difference spectra of cytochromes c and c_1, Fig. 3, on the basis of their respective potentiometric properties, we analysed series of titration curves from the data base shown in Fig. 1 B with the E_m parameters kept constant at values reported above. This E_m parameter is not wavelength dependent for a given cytochrome and here again, we could not justify including uncertainties on this parameter when resolving individual difference spectra. The peak positions of the resolved α bands as well as of their sum (Fig. 3) correspond to the ones determined in these mitochondria where cytochrome c could be salt extracted

(Ducet et al. 1970). The ratio (2/1) of the respective contributions of cytochromes *c* and c_1 to the α band of the difference spectrum (Fig. 3) is in reasonable agreement with the earlier estimation (1/1) of Ducet et al. (1970). As outlined in Materials and methods (Data analysis), the fitted parameter DA_j is composed of the product $l\,C_j\,\Delta\varepsilon_{j_{ox-red}}$ where *l*, the optical pathlength, is usually defined with high accuracy. If one of the other terms, concentration or difference molecular extinction coefficient, is known independently, the fitted parameters DA_j can be further worked out to determine the other term. This independent information being not available for *c*-type cytochromes in potato tuber mitochondria, we did not extend our analysis of the individual optical contributions as resolved in Fig. 3. Nevertheless, very useful information could be provided for other complex systems through this possibility.

Other approaches have been developed to simultaneously resolve potentiometric and spectral properties of redox compounds in complex systems. One is based on a combined spectrum deconvolution and potentiometric analysis (Hendler and Shrager 1979; Van Wielink et al. 1982). We consider this approach very questionable as it brings together two unrelated analysis procedures. Indeed there is no direct relationship between redox potential and analytical restitution of a complex optical profile by a series of gaussian curves for instance. There is no physical meaning in linking spectra second derivative or height of a gaussian component to the redox potential of the suspension (Hendler and Shrager 1979). Such gaussian components cannot be identified with specific absorbing species (see for instance Denis and Deyrieux 1977). The other approach, labelled SVD for singular value decomposition, has been proposed by Shrager and Hendler (1982). In our view, this approach suffers from the same kind of misleading combination of unrelated analysis procedures. In this method, a set of spectra like the one in Fig. 1 B is replaced by *r* linearly independent profiles which play the role of the *r* redox species of the system under investigation. This step is perfectly valid even if it is exposed to several limitations (Denis and Deyrieux 1977). However, there is no physical meaning in linking these *r* spectra with the redox potential values of the experimental titration. No solution can be expected through this approach since the linear spectra combinations, from which the *r* profiles are derived, cannot be applied to the measured redox potential values, the relationship between absorbance and redox potential being a non-linear one (see Eq. (1)). The misleading association of two unrelated analysis procedures are to be considered as responsible of the unrealistic resolution of cytochrome c_1 into 2 or even 3 compounds, all with $n=2$ (Reddy and Hendler 1986).

In our study, we did not address the *b*-type cytochromes nor cytochrome *c* oxidase. Both hemes *b* and at least heme a_3 belong to the membraneous compartment, preventing application of the Nernst relationship to analysing titration curves built with redox potentials measured in the aqueous phase and redox states derived from within the membrane by spectrometry (see Walz 1979). It is worth remembering that the Nernst equation is only valid for single phase systems. Interactions between redox centers belonging to the same protein, as in cytochrome *c* oxidase (Blair et al. 1986) make the situation even more complex. It is then necessary to rely on a formalism which complies with thermodynamic principles (Walz 1979). Using such a formalism, Walz and Denis (1984) derived a new model to analyse redox titrations of hemes *b* in mitochondrial complex III. The experimental application of this approach remains to be done. This is a basic problem which has to be solved to open the way, at last, to reliable potentiometric investigations on membraneous redox components.

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