INTERFERENCE BY CYTOCHROMES IN THE MEASUREMENT OF CALCIUM WITH MUREXIDE

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1. Introduction

The calcium-binding dye murexide has become a widely-used tool for measuring changes in the free Ca^{2+} concentration in biological systems. The method was introduced in this context in [1,2] and the present status of the measurements, together with certain problems encountered in the use of the dye, was the subject of the review [3].

Murexide was first used to study mitochondrial Ca^{2+} accumulation in [4] where calcium- dependent colour changes of the dye in suspensions of rat liver mitochondria were observed with a dual wavelength spectrophotometer. The wavelength pair 540–510 nm was employed for the murexide measurements [4]. Here, we show that, if this wavelength pair is used with mitochondrial suspensions, the contribution to the difference signal from changes in the redox poise of the cytochromes is incompletely eliminated.

Since the original mitochondrial experiments [4], optical measurements have been made with murexide, of the free calcium concentration in mitochondrial suspensions (e.g., [5–8] and the reviews in [9,10]). In [5–10] the wavelength pair 540–507 nm has generally been used. Our measurements show that although 540 nm is almost isosbestic for the cytochrome system during the aerobic to anaerobic transition, 510 nm (which is almost isosbestic for the calcium-induced spectral change of murexide) is not adequately isosbestic for this redox change in the cytochrome system. A dual wavelength instrument was employed in [4] which used interference filters of comparatively large bandwidth, and this may have largely obliterated the interference described here.

The problem of interference by cytochromes became apparent to us during experiments on pig skeletal muscle mitochondria. Various breeds of pig differ markedly in their susceptibility to stress and differences have been demonstrated [11–13] to exist between mitochondria isolated from these various breeds. Some of the experimental observations in [11–13] involved the use of the murexide method and the wavelength pair 540–510 nm to monitor respiration-dependent calcium accumulation and retention by the mitochondria. When the suspension became anaerobic, the resultant loss of intra-mitochondrial calcium was monitored. This calcium efflux appeared to be kinetically complex, with an early fast phase which was succeeded by a readily distinguishable later slower phase. Both the fast phase and the later slower phase were faster in mitochondria isolated from stress prone animals [11]. We now believe that the early fast phase at the onset of anaerobiosis, attributed to calcium efflux [11–13] is actually contributed by the reduction of *c*-type cytochromes.

2. Materials and methods

We employed two spectrophotometers. Both instruments were operated in the dual wavelength mode. Instrument A was built around two Heath E U 700-E monochromators which were operated with a slitwidth which provided a spectral bandwidth of 2.8 nm ('measuring' channel) and 2.2 nm ('reference' channel) during these experiments. The instrument output was smoothed by an RC time constant of 0.1 s, and the output from the measuring channel was

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone; EGTA, ethyleneglycol bis (β-aminoethyl ether) *N*,*N'*-tetraacetic acid

displayed (after a suitable offset voltage had been applied) as a single-ended signal, together with the dual wavelength difference signal, on the two channels of a potentiometric strip chart recorder (J.J. Instruments C R 552). Instrument B was an Aminco DW 1 spectrophotometer operated with an output time constant of 0.4 s except for the 540–550 nm measurements of fig.5, when the time constant was reduced to 0.1 s. The spectral bandwidth employed with this instrument was 2.8 nm for both 'measuring' and 'reference' channels. Instrument A was employed for the experiment recorded in fig.1, and instrument B was used for the rest of the experiments. All experiments were conducted at 23°C.

Pig skeletal muscle mitochondria were isolated, after limited proteolysis of the muscle, in the manner of [14] and spectroscopic experiments were conducted in a 1 cm pathlength cuvette as in [11] in a medium containing 225 mM mannitol, 75 mM sucrose and 20 mM Tris–HCl (pH 7.2). Protein was determined by the Lowry method [15] using bovine serum albumin as standard. Rotenone, antimycin A, and FCCP were added in small volumes of ethanol. Less than 10 μl of ethanol was added in the course of an experiment.

The experiments were started by the addition, to the aerobic rotenone-inhibited mitochondrial suspension, of $CaCl_2$ to 200 μM final conc., followed by succinate (Na-salt) (7.3 mM) (final suspension vol. 2.8 ml) to produce respiration-dependent calcium accumulation. Net calcium accumulation finally ceased and then, when oxygen was exhausted, net calcium efflux occurred. After a short anaerobic period the suspension was aerated by gentle shaking and the subsequent optical events were recorded. In this way, the aerobic to anaerobic transition, and the consequences of calcium accumulation and loss, could be studied repeatedly in a single mitochondrial suspension. In the presence of added Ca^{2+}, the first aerobic to anaerobic transition in an experimental series observed at 540–510 nm, was seen to be slightly different from subsequent ones which were identical to one another. The first such transition was therefore not employed for comparison purposes.

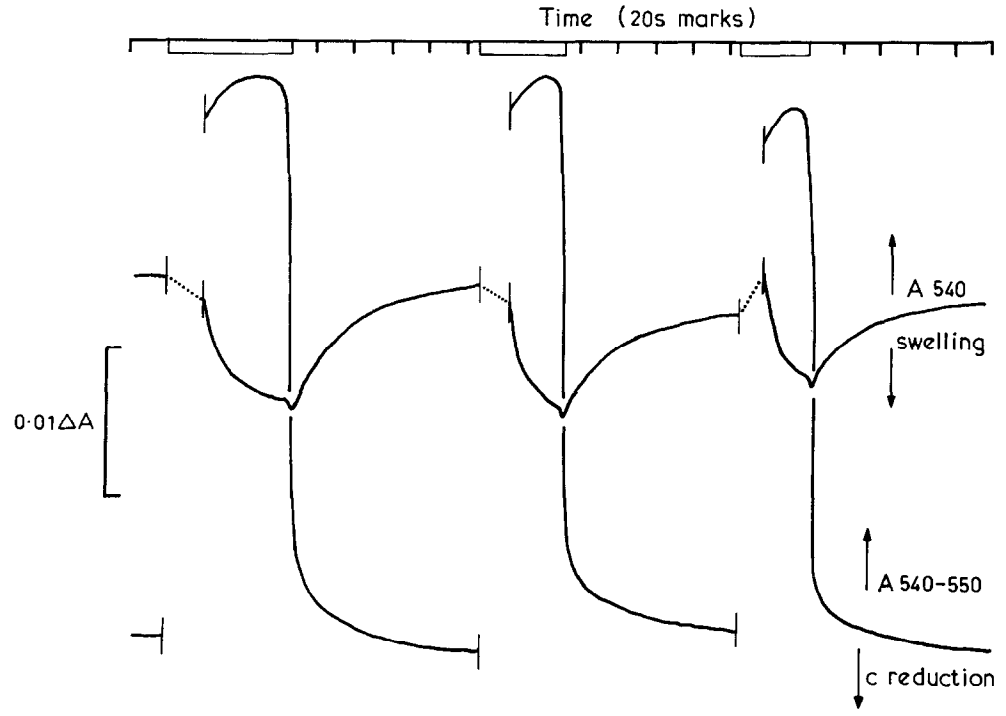

Fig.1. Light scattering and ΔA_{540} associated with the alternation of aerobic and anaerobic states. Mitochondria (2.8 mg protein/ml) were provided with rotenone (1.7 μg/ml) calcium and succinate and allowed to go anaerobic in the dual wavelength spectrophotometer. $\Delta A_{540-550}$ and ΔA_{540} are recorded which resulted from 3 successive aerobic periods (indicated by the 3 bars at the top of the figure). The records are interrupted for the intervals when the suspension was shaken.

An unusual convention has been adopted for displaying these dual wavelength measurements. The monochromator of the 'measuring' channel was always set at 540 nm, an isosbestic wavelength for the cytochrome changes, whilst the wavelength supplied by the 'reference' channel was changed. Adherence to this convention facilitates comparison of the various absorption changes (ΔA) with those results obtained at a wavelength pair regarded as appropriate for measuring the calcium-dependent colour changes of murexide. The convention does, however, cause changes in the recorded ΔA to be in a direction which is the reverse of that normally encountered. In all the figures, absorbance increase is upwards.

3. Results and discussion

Figure 1 presents the results of an experiment using the calcium-accumulating protocol of [11] but in the absence of murexide. Simultaneous measurements were made of the redox state of cytochrome $c + c_1$ at 540–550 nm (to monitor the onset of the anaerobic state) and also of A_{540} alone. The suspension was rendered aerobic at intervals as indicated. It is evident that at 540 nm the main ΔA is a gradual increase in the anaerobic state and a gradual decrease in the aerobic state. This may be attributed to the alternate (low amplitude) shrinking and respiration-dependent swelling of the mitochondria [16]. A small but rapid absorbance decrease is apparent at the time of onset of the anaerobic state. The amplitude of this rapid change varies somewhat depending upon the time for which the mitochondrial suspension has been aerobic. It may represent a contribution from *c*-type cytochromes. Measurements made in the dual wavelength mode, and conducted according to [11] are recorded in fig.2. Murexide was present and repetitive re-aeration of the suspension permitted the examination of a range of 'reference' wavelengths during the aerobic to anaerobic transition. It is apparent that at 510 nm there is a substantial early fast decrease in absorbance before the later slower phase of calcium efflux is seen. Analysis of this secondary slow phase of absorbance decrease (not shown) shows that it exhibits first order kinetics. The early fast phase is made smaller when the 'reference' wavelength is decreased. At shorter wavelengths, however, an early ΔA is seen which is kinetically rather complex. This change is no longer monotonic, an early decrease in

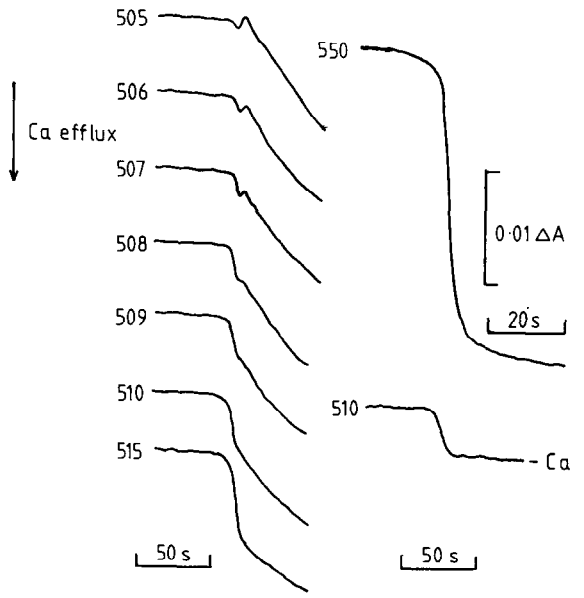

Fig.2. Wavelength-dependence of the ΔA which ensue in the presence of murexide when the mitochondria become anaerobic. Mitochondria (2.3 mg protein/ml) supplemented with rotenone, (1.7 μg/ml), murexide 92 μM, calcium and succinate, were observed with the wavelength pair 540–510 nm and allowed to go anaerobic. At intervals of ~100 s, the suspension was made aerobic and the observations were repeated at various 'reference' wavelengths as indicated. An $A_{540-510}$ decrease of 0.007 results from the addition of 100 ng-ions Ca^{2+} to the cuvette. EGTA (300 μM) was then added and the record labelled (–Ca) was obtained at 540–510 nm.

absorbance is succeeded by a later increase, before the steadily decreasing absorbance characteristic of calcium efflux begins to predominate. At wavelengths <506 nm, the early fast absorbance decrease becomes an increase in absorbance.

The experiment labelled (–Ca) in fig.2 was conducted after the addition of 0.3 mM EGTA sufficient to lower free Ca^{2+} outside the mitochondria to $<10^{-6}$ M. The change attributable to steady calcium efflux is lost but the rapid absorbance decrease remains. The EGTA addition does slow the fast change, possibly by retarding the decay of the high energy state [17,18].

The experiments of fig.3 were conducted in a fashion similar to those of fig.2 but in the absence of murexide, and the spectral changes which occur independently of calcium binding by the extra mitochondrial dye are now more clearly apparent. The ΔA observed in the aerobic–anaerobic transition, at

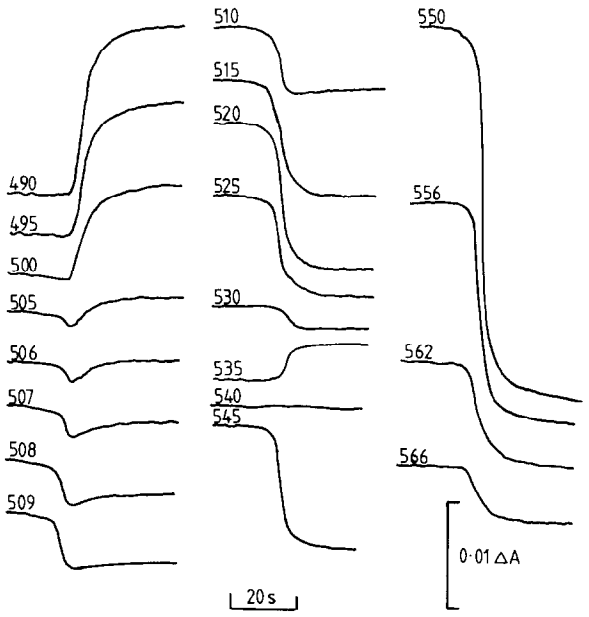

Fig.3. Wavelength dependence of the ΔA which ensue in the absence of murexide. A mitochondrial suspension (2.9 mg protein/ml) was treated as in fig.2, except that the murexide was omitted. The mitochondria were repeatedly rendered aerobic as in the experiment of fig.2 and absorbance measurements were made, as the suspension became anaerobic, at a range of 'reference' wavelengths as shown.

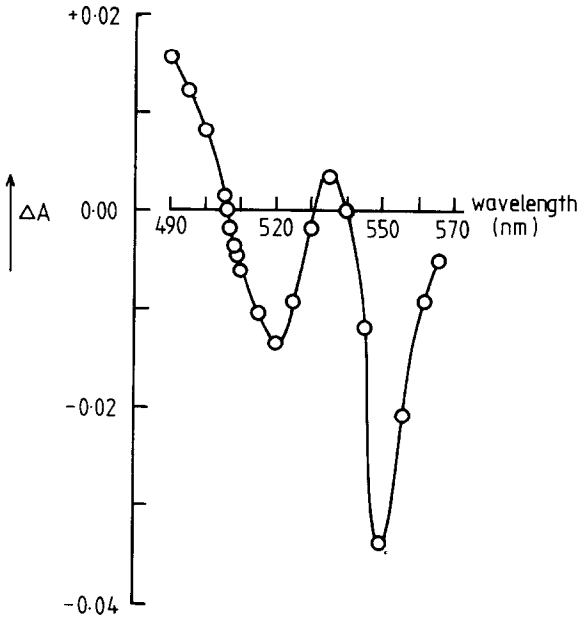

Fig.4. A steady state-oxidised minus fully substrate-reduced difference spectrum compiled from the experiments of fig.3, by measuring the amplitude of the ΔA between a point on the absorbance trace, 7 s before the first upward or downward inflection, and a point 14 s after that point.

wavelengths <510 nm, is not monotonic and an early fast decrease is succeeded by a later slower increase in absorbance.

The β absorption peak of cytochrome *c* (521 nm) and that of cytochrome *b* (532 nm) are separated by 11 nm [19] and so it seems plausible that the respective isosbestic points for these cytochromes, on the short wavelength side of this peak in the region of 510 nm, should be separated by a similar distance. Isolated horse-heart cytochrome *c* exhibits an isosbestic point at 504 nm [20]. It appears likely, therefore, that there is a wavelength region around 510 nm which is on opposite sides of the isosbestic points of the *c*- and *b*-type cytochromes so that in this region the transition from steady state-oxidised to fully substrate-reduced, causes absorbance excursions of opposite sign to be contributed by the *c*- and *b*-type cytochromes, respectively. The slower rates of reduction of the cytochromes *b* (measured at 540–562 nm and 540–566 nm) compared with the cytochromes $c + c_1$ measured at 540–550 nm (see fig.3) are also consistent with the interpretation of the early fast decline in absorbance as due largely to cytochromes $c + c_1$ and of the later slower rise as due to cytochromes of *b*-type.

In fig.4 is presented a spectrum, compiled from fig.3, of the overall absorbance change in the aerobic to anaerobic transition from a wavelength of 490–570 nm. The functional spectrum thereby established is characteristic of the β and α absorption bands of cytochromes $c + c_1$ with a small additional contribution from the *b*-type cytochromes. This result is consistent with the expectation that the degree of reduction of cytochromes *b* should already be substantially greater than that of cytochromes of *c*-type during the steady state-oxidised condition in the presence of calcium. Other measurements made in the absence of murexide at 540–550 nm (cytochromes $c + c_1$) of the effect of adding antimycin A (0.3 μg/mg protein) plus FCCP (0.5 μM) to establish practically complete oxidation of *c*-type cytochromes, indicated that in the oxidised steady state in the presence of added Ca^{2+}, *c*-type cytochromes are 20% as reduced as in the fully substrate-reduced condition. In the case of *b*-type cytochromes, measured at 540–562 nm, full oxidation with ferricyanide (0.5 mM) and FCCP

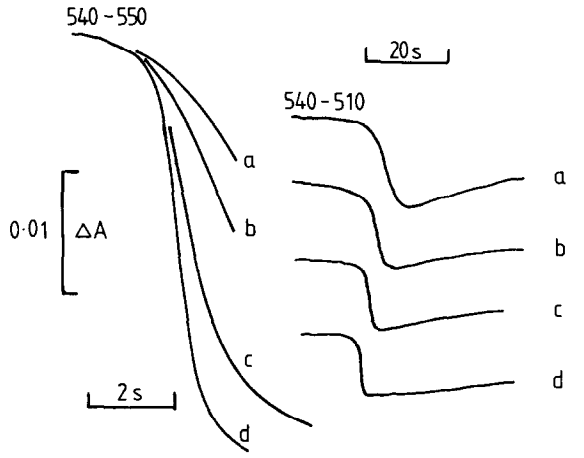

Fig.5. The effect of FCCP upon the rate of reduction of cytochromes $c + c_1$ and upon the kinetics of the murexide-independent $\Delta A_{540-510}$. A mitochondrial suspension (2.9 mg protein/ml) was prepared as described in fig.2 except that both calcium and murexide additions were omitted. The mitochondria, rendered anaerobic by respiratory activity, were made aerobic repeatedly and dual wavelength records were made as the suspension became anaerobic at both 540–510 nm and at 540–550 nm in the presence of various FCCP concentrations as indicated by labels against the traces: (a) none; (b) 1×10^{-8} M; (c) 2×10^{-8} M; (d) 4×10^{-8} M.

(0.5 μM), added in the absence of succinate, together with our other measurements at this wavelength pair, indicated that in the oxidised steady state characteristic of these present experiments, *b*-type cytochromes are 60% as reduced as in the fully substrate-reduced state.

The data recorded in fig.5, in the absence of added calcium or murexide, indicate the effect upon $A_{540-510}$ and $A_{540-550}$, of the addition of very low concentrations of the classical uncoupling agent FCCP. The progressive increase in the uncoupler concentration would be expected to increase progressively the rate of decay of the protonmotive force upon the onset of anaerobic conditions and to increase the rate of electron flow through the respiratory chain [18]. It can be seen that the uncoupler-dependent acceleration of the rate of cytochrome $c + c_1$ reduction measured at 540–550 nm is rather closely similar to the acceleration of the early fast $A_{540-510}$ decrease which is produced by the uncoupling agent. It can also be seen that the later slow rise in $A_{540-510}$ is progressively reduced in amplitude. This latter effect appears likely to be due to a progressively increased amplitude and faster rate of contribution from the reduction of cytochromes *b*, since measurements (not shown) at 540–562 nm indicated that the redox poise of the *b*-type cytochromes in the steady state oxidised condition was indeed shifted in the oxidised direction by such additions of FCCP and that the rate of cytochrome *b*-reduction was also accelerated.

Comparison of fig.3 with fig.5 indicates that the addition of Ca^{2+} has been an effect at 540–510 nm rather similar to the effect of adding a very low concentration of classical uncoupling agent. Further work is in progress to clarify this feature in the light of the differences which have been observed between the kinetics of cytochrome reduction in mitochondria isolated from pigs which differ in their susceptibility to stress.

4. Conclusions

These experiments emphasize the difficulty of making high resolution optical measurements of free calcium concentration with the dye murexide when metabolically-active mitochondria are present. The selection of a suitable wavelength pair is critical, particularly in the case of experiments which involve aerobic–anaerobic transitions, because of the large change in the redox poise of the *c*-type cytochromes which accompanies this transition. Moreover, in these skeletal muscle mitochondria, the cytochrome *c* concentration is high (~0.5 μmol/g protein) [21] when compared with the rat liver mitochondrial level of ~0.2 μmol/g protein [17], and this increases the experimental difficulty which is encountered.

The early fast phase of calcium efflux noted in [11–13] is largely accounted for by the ΔA of the *c*-type cytochromes, and our measurements indicate that the later slower phase, if observed at >30 s after the onset of the anaerobic state, is uncontaminated by redox changes of the cytochromes and presumably can be attributed to the loss of calcium from the mitochondrial matrix space.

Measurements made with a Ca^{2+} specific electrode (data not shown) also indicate no early fast phase of calcium efflux when the mitochondria go anaerobic, but only a persistent slow loss which exhibits first order kinetics identical with those seen in the later stages of murexide measurements.

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