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THE CYTOCHROME *b* SPECTRUM OF PLANT MITOCHONDRIA AND ITS RESPONSE TO ATP AND ANTIMYCIN

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SUMMARY

1. In Jerusalem-artichoke submitochondrial particles four *b*-type cytochromes are shown in spectra recorded at different redox potentials at 25 °C, one of which is reducible only by $Na_2S_2O_4$ at lower potentials ($Na_2S_2O_4$-reducible *b*).

2. The addition of ATP to cauliflower mitochondria in State 3 or 5 causes an increase in reduction of the three succinate-reducible *b* cytochromes.

3. Exogenously added ATP has no effect on the *b* spectrum of artichoke mitochondria, although cytochrome *b* is more reduced in State 4 than in either State 3 or the uncoupled state.

4. The addition of antimycin to plant mitochondria under aerobic conditions or together with oxygen after anaerobiosis results in an increased reduction of three succinate-reducible *b* cytochromes.

5. Antimycin also induces a red-shift in the spectrum of $Na_2S_2O_4$-reduced mitochondria, the magnitude of which is less than that observed in animal mitochondria.

INTRODUCTION

An early indication of the presence of multiple mitochondrial *b*-type cytochromes was the demonstration by Bonner[1–3] of three clearly-defined absorption maxima at 553, 557 and 562 nm at 77 °K in plant mitochondria. However, although kinetic[4] and potentiometric[5] measurements have distinguished three *b*-components at room temperature, generally at 25 °C the α-band of cytochrome *b* appears as a single broad maximum centred around 560 nm, and it is still unclear at which wavelengths the absorption maxima of the individual components occur.

Following the realization that also in animal mitochondria there are several *b*-type cytochromes, considerable interest has been focussed on the number of components present, and whether any of these components may be directly involved in oxidative phosphorylation[6–11]. Disagreement still exists as to whether the maxima observed at 558 and 565 nm at 25 °C in animal mitochondria represent two separate

Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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cytochromes or the split α-band of a single cytochrome[9–11], and concerning the number of *b*-components that are affected by ATP[7,12,13].

Recently, we have found that it is also possible at room temperature to distinguish spectrally three cytochrome *b* peaks in plant mitochondria and submitochondrial particles. Since the wavelength span between the peaks is slightly greater, the absorption maxima at 556, 560 and 566 nm are rather more readily resolved than in beef-heart mitochondria. In view of this fact, we have utilized plant mitochondria to demonstrate that each peak represents a single cytochrome, and to investigate which cytochromes are affected by ATP. In addition, we have investigated further the effect of antimycin on the *b* spectrum in order to settle the question left open by Bonner and Slater[14] as to whether or not there is an antimycin-induced spectral shift in plant mitochondria.

METHODS

Mitochondria were prepared from Jerusalem-artichoke tubers or cauliflower buds following methods previously described[15,16], except that the cauliflower tissue was ground for approx. 5 s with an Ultra-Turrax blendor, and 10 mM Tris–HCl buffer (pH 7.4) was included in the isolation medium. Submitochondrial particles were prepared by sonication of artichoke mitochondria that had been previously frozen and thawed. Difference spectra at 25 °C or 77 °K were measured using either an Aminco-Chance or a Perkin–Elmer 356 double-beam, dual-wavelength spectrophotometer. Potentiometric redox titrations were carried out anaerobically in a closed cuvette fitted with a platinum electrode and a calomel reference electrode and connected to an Aminco-Chance dual-wavelength spectrophotometer. The solution was bubbled with N_2 prior to closing the cuvette, and N_2 was passed over the surface of the medium throughout the experiment. Spectra were recorded at the desired potentials.

Following the recommendation of the enzyme nomenclature commission[17], and in accordance with general practice in animal mitochondria, the cytochromes here will be referred to with suffixes representing the absorption maxima at room temperature.

RESULTS

The presence of three distinct *b*-type cytochromes in Jerusalem-artichoke submitochondrial particles is demonstrated in Fig. 1, which records spectra at different potentials during the course of a potentiometric redox titration, beginning at 340 mV and decreasing the potential stepwise until −100 mV by addition of NADH and $Na_2S_2O_4$. It is clear that between 340 and 210 mV solely cytochromes *c* and c_1 are reduced. Cytochrome *b*-556 is mainly reduced between 100 and 50 mV, cytochrome *b*-560 is reduced between 100 and 20 mV, while cytochrome *b*-566 does not become reduced until between 20 and −50 mV. An additional component with a peak at 563 nm and a shoulder between 559 and 561 nm appears between −50 and −100 mV. This represents $Na_2S_2O_4$-reducible cytochrome *b* which may be denatured and is probably not of physiological importance, because it was present in larger amounts in particles than in intact mitochondria. In this experiment submitochondrial particles

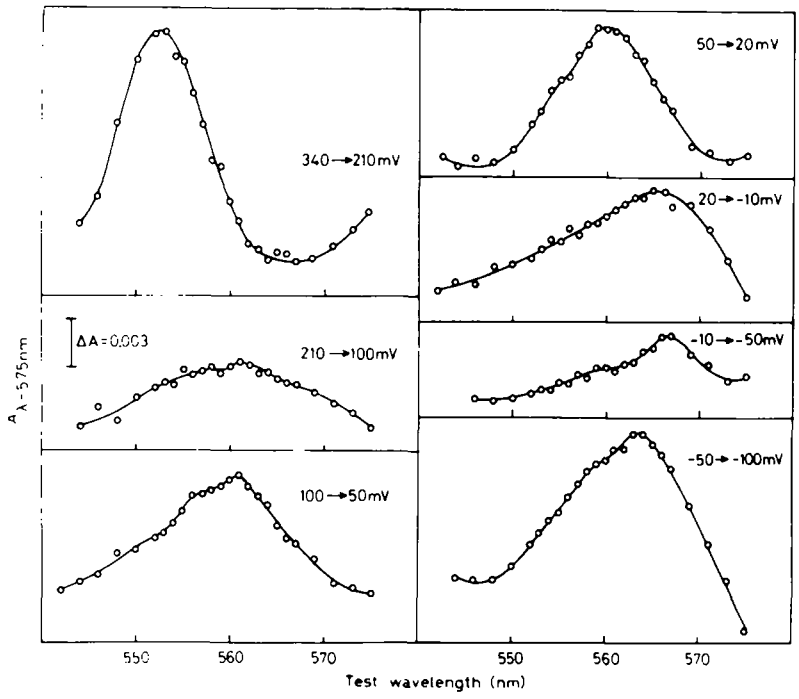

Fig. 1. Difference spectra of the *b*-type cytochromes in Jerusalem-artichoke submitochondrial particles reduced at different redox potentials during a potentiometric redox titration. Submitochondrial particles (3 mg protein/ml) were suspended in a medium containing 0.4 M sucrose, 0.03 M Tris–HCl buffer (pH 7.2), 1 μM FCCP, 30 μM diaminodurene, 20 μM phenazine ethosulphate, 20 μM phenazine methosulphate, 50 μM duroquinone, 4 μM pyocyanine, 25 μM 2-hydroxy-1,4-naphthoquinone and 25 μM anthraquinone-1,5-disulphonate. The potential of the system was made more positive by the addition of ferricyanide, and a reductive titration was performed by the gradual addition of small amounts of 100 mM NADH, and for the lower potentials freshly-prepared aqueous $Na_2S_2O_4$. Spectra were recorded at the indicated redox potentials. After the titration, ferricyanide was again added and the reversibility of the titration confirmed. During the course of the titration a reversible, redox-dependent change in an unidentified component caused a change in the baseline between 200 and 50 mV. The spectra here were corrected for this baseline alteration.

were used rather than intact mitochondria, because in the absence of endogenous substrate it was easier to maintain a constant potential during spectral measurements.

The effect of energization on the cytochrome *b* spectrum of plant mitochondria is shown in Fig. 2. The addition of ATP to succinate-reduced cauliflower mitochondria at anaerobiosis causes in increase in absorption at 565–575 nm. The ATP *minus* carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) difference spectrum shows an absorption maximum in the energized state at 567 nm at 25 °C. At 77 °K (Fig. 3) this peak splits to reveal the reduction of all three *b* components. Cytochrome *c* is partly oxidized indicating a reversal of electron flow in the presence of ATP. The addition of ATP to artichoke mitochondria, however, has no effect on the cytochrome *b* spectrum under either aerobic or anaerobic conditions. An effect of energization may nevertheless be seen in artichoke mitochondria during the

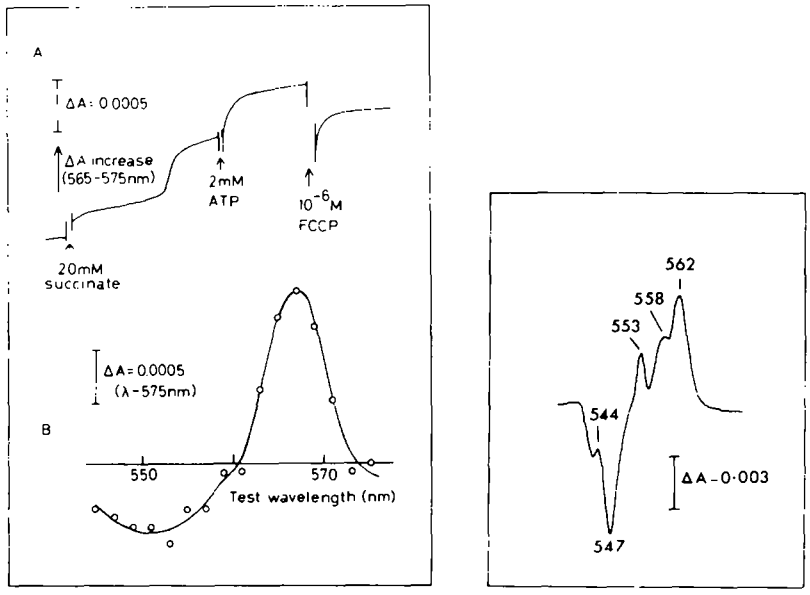

Fig. 2. The effect of ATP on the cytochrome *b* spectrum of cauliflower mitochondria. The cuvette contained 0.3 M mannitol, 10 mM Tris–HCl buffer (pH 7.2), 1 mM EDTA, and mitochondria (6 mg protein/ml), at 25 °C. Other additions were made as indicated. A, changes in absorbance monitored at 565–575 nm; B, difference spectrum, ATP anaerobic *minus* FCCP anaerobic.

Fig. 3. The effect of ATP on the cytochrome *b* spectrum of cauliflower mitochondria. The cuvettes contained 0.3 M mannitol, 10 mM Tris–HCl buffer (pH 7.2), 1 mM EDTA, 10 mM succinate, 2 mM ATP and mitochondria (3 mg protein/ml). To the reference cuvette was added 1 μM FCCP, and after anaerobiosis the difference spectrum was recorded at 77 °K.

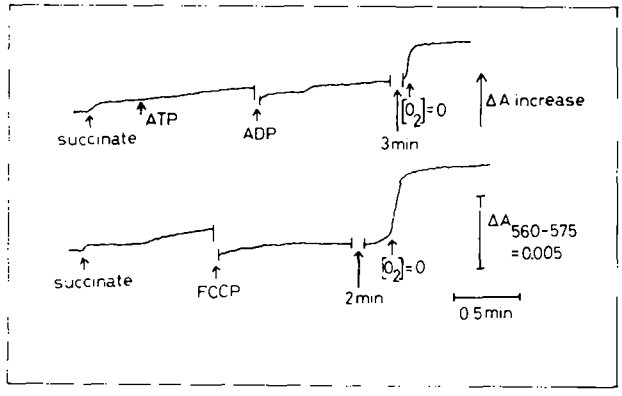

Fig. 4. The effect of energization on the level of cytochrome *b* reduction in Jerusalem-artichoke mitochondria. The cuvette contained mitochondria (2.4 mg protein/ml) suspended in a medium consisting of 0.4 M sucrose, 0.035 M Tris–HCl buffer (pH 7.2), 1 mM EDTA and 10 mM phosphate and absorption changes were monitored at 560–575 nm. Further additions were made as indicated: 8 mM succinate, 1 μM FCCP, 0.7 mM ATP and 0.2 mM ADP.

State 4 to State 3 transition, or on addition of uncoupler to mitochondria in State 4. These experiments are illustrated in Fig. 4. The addition of ADP to artichoke mitochondria oxidizing succinate slightly decreases the absorption at 560–575 nm, which returns to the original level, presumably after exhaustion of the ADP, prior to anaerobiosis. The addition of FCCP to aerobic mitochondria in State 4 decreases the absorption at 560–575 nm which does not increase until anaerobiosis. Difference spectra at 77 °K, shown in Fig. 5, indicate that in the energized state a crossover[18] at Site 3 influences the level of cytochrome reduction. Thus in the aerobic state (Fig.5A) there is an increased reduction during energization of the *b*-type cytochromes, with absorption maxima at 553, 559 and 562 nm (at 77 °K), and of cytochrome *c* with a maximum at 548 nm. At anaerobiosis (Fig. 5B) the cytochromes *b* are more reduced (peaks at 553, 558 and 562 nm) and cytochrome *c* more oxidized (trough at 547.5 nm) under energized conditions, corresponding to a reversal of electron flow. The addition

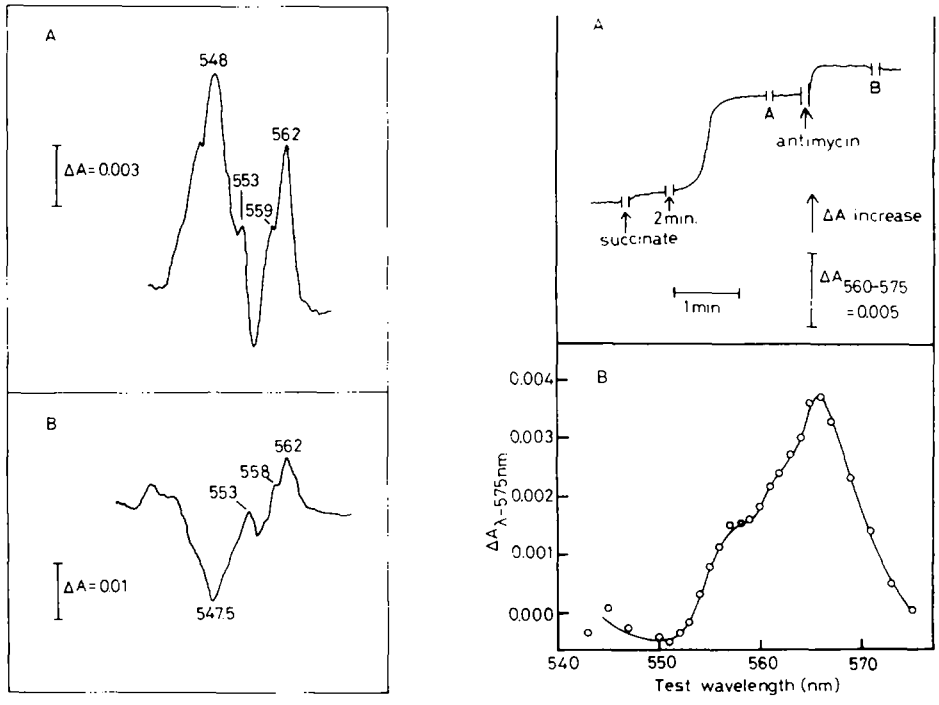

Fig. 5. The effect of energization on the cytochrome *b* spectrum of Jerusalem-artichoke mitochondria. Spectra were measured at 77 °K. The cuvettes contained 0.24 M sucrose, 0.035 M Tris–HCl buffer (pH 7.2), 8 mM succinate and mitochondria (8 mg protein/ml). To the reference cuvette was added 1 μM FCCP. A, difference spectrum under aerobic conditions; B, difference spectrum after anaerobiosis.

Fig. 6. The effect of antimycin on the cytochrome *b* spectrum of substrate-reduced Jerusalem-artichoke mitochondria. The cuvette contained 0.4 M sucrose, 0.035 M Tris–HCl buffer (pH 7.2), 1 mM EDTA, 1 μM FCCP and mitochondria (2.4 mg protein/ml), at 25 °C. The reaction was started with 8 mM succinate. Antimycin (0.5 nmole/mg protein) was added where indicated, and O_2 was stirred in at the same time. A, absorption changes monitored at 560–575 nm; B, difference spectrum with 575 nm as reference wavelength, anaerobic + antimycin (B in Fig. A) *minus* anaerobic (A in Fig. A).

of oligomycin to these mitochondria in the presence of ADP and P_i also increases the reduction of cytochrome *b*. Similar effects of energization on the *b* spectrum may by observed in cauliflower mitochondria.

As shown in Fig. 6A, the addition of antimycin (together with oxygen) to Jerusalem-artichoke mitochondria, made anaerobic with succinate in the presence of FCCP, induces an increase in absorption at 560–575 nm. The difference spectrum obtained by subtracting the spectrum at anaerobiosis in the absence of antimycin (A in Fig. 6A) from that in the presence of antimycin (B in Fig. 6A) is illustrated in Fig. 6B. An absorption maximum at 566 nm is observed, with shoulders at 562–563 nm and 557–558 nm, indicating that all three *b* cytochromes are affected. Fig. 7 shows that at 77 °K the three components are more clearly identified, with maxima at 553.5, 558.5 and 563 nm. Fig. 7 was obtained using cauliflower mitochondria, which respond to antimycin in a similar way as to artichoke mitochondria. Cauliflower mitochondria, unlike artichoke mitochondria, possess in addition to the normal cyanide-sensitive respiration mediated by cytochrom *c* oxidase a cyanide-insensitive respiration. Thus, the effect of antimycin on the *b* spectrum appears to be independent of the presence or absence of a cyanide-insensitive pathway.

The increased reduction brought about by the addition of antimycin after anaerobiosis is greatest for *b*-556; *b*-560 and *b*-556 are affected to a lesser extent. Under uncoupled, aerobic conditions, where *b*-560 and *b*-556 are also highly oxidized, antimycin addition causes considerable reduction of all three *b* cytochromes. In order to determine whether the increase in absorbance is due solely to a change in

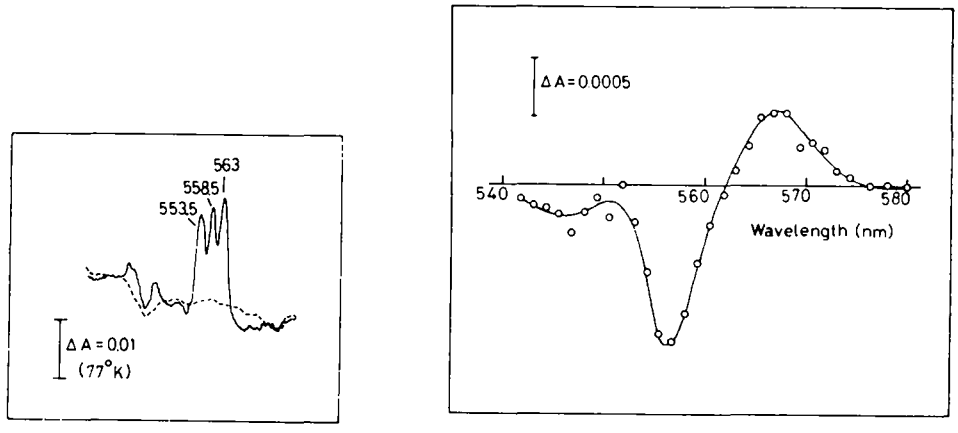

Fig. 7. The effect of antimycin on the cytochrome *b* spectrum of succinate-reduced cauliflower mitochondria, measured at 77 °K. The cuvettes contained 0.3 M mannitol, 10 mM Tris–HCl buffer (pH 7.2), 1 mM EDTA, 8 mM succinate, 1 μM FCCP and mitochondria (5 mg protein/ml). After anaerobiosis, antimycin (1 nmole/mg protein) together with air was stirred into the sample cuvette. The difference spectrum, antimycin *minus* anaerobic, taken 10 min after antimycin addition, is shown by the solid line. The broken line represents the baseline, without antimycin in the sample cuvette.

Fig. 8. The antimycin-induced shift in the cytochrome *b* spectrum of $Na_2S_2O_4$-reduced Jerusalem-artichoke mitochondria. The sample and reference cuvettes contained mitochondria (6 mg protein/ml) reduced with $Na_2S_2O_4$ and suspended in a medium containing 0.4 M sucrose, 0.035 M Tris–HCl buffer (pH 7.2) and 1 mM EDTA. Antimycin (0.5 nmole/mg protein) was added to the sample cuvette. A baseline, prior to the addition of antimycin, was subtracted from the difference spectrum.

the redox state, as was indicated for mung-bean mitochondria by Storey[19], or also to a spectral shift[16,20,21], the effect of antimycin was investigated under conditions where all the *b*-cytochromes in artichoke mitochondria were previously reduced with $Na_2S_2O_4$. Fig. 8 indicates that antimycin brings about an increase in absorption at 567 nm and a decrease at 557 nm, corresponding to a shift in the spectrum. Subsequent addition of antimycin to the reference cuvette abolishes the effect. The absorption maximum at 567 nm and the minimum at 557 nm may indicate that the shifting component is *b*-560. Making this assumption, the magnitude of the shift ($\Delta A_{peak-trough}$) expressed as a percentage of the total ΔA of cytochrome *b*-560 is less than half that found in beef-heart submitochondrial particles (22%, refs 21, 22).

DISCUSSION

We conclude that the three components in artichoke submitochondrial particles reduced at different potentials between 210 and -50 mV represent three *b* cytochromes, thus providing spectral confirmation of the earlier kinetic and potentiometric data of Storey[4,5]. There is no indication that *b*-556 and *b*-566 are the split α-band of a single hemochrome. However, the relevance of this finding to the situation in animal mitochondria is still uncertain. It is possible that *b*-556 is not present in animal mitochondria and that in plant mitochondria it obscures a shoulder at 558 nm present in both plant and animal mitochondria.

Dutton and Storey[5], using mung-bean mitochondria, have shown the midpoint potentials of cytochromes *b*-556, *b*-560 and *b*-566 to be 75, 42 and -77 mV, respectively, at pH 7.2. We have calculated the E'_0 values for the *b*-566 and *b*-560 to be 75 and 40 mV, respectively, in submitochondrial particles, but we find the E'_0 of *b*-566 to be 0 mV, if correction is made for the $Na_2S_2O_4$-reducible component ($E'_0 = -90$ mV), which contributes at the lower potentials. If no correction is applied we then find a current E'_0 of -70 mV for *b*-566. Lance and Bonner[3] have shown $Na_2S_2O_4$-reducible *b* to be present in considerable amount in intact microchondrial preparations, and it may also be necessary here to correct for its contribution.

The increase in reduction of all *b* cytochromes on addition of ATP to cauliflower mitochondria is in agreement with the conclusions of Berden *et al.*[12] and Pedersen and Flatmark[13], with respect to animal mitochondria, but in contrast to the conclusions of Chance *et al.*[7]. The failure of ATP to induce any change in the *b* spectrum of artichoke mitochondria, although effects of energization may be observed during changes in respiratory state, probably reflects the unusual adenine nucleotide translocation properties of these mitochondria[23]. Artichoke mitochondria have previously been shown to lack an uncoupler-induced ATPase activity, even though oxidative phosphorylation may be effectively uncoupled from electron transport[16]. The present results further emphasize the inability of the ATPase (F_1) to hydrolyse exogenously added ATP in the intact mitochondria, indicating possibly a close similarity to the apparent lack of an effect of exogenous ATP in mitochondria from *Candida utilis*[24].

In agreement with Storey[19], we conclude that the principal effect of antimycin on the *b* spectrum of succinate-reduced plant mitochondria is an increased reduction of cytochrome *b*. We find that antimycin affects all three *b* cytochromes (similar to the results described for animal mitochondria[25]) with a maximum increase at

566 nm. The greater increase in reduction of *b*-566 compared with *b*-560, rather than a spectral shift, would account for the increase in absorption at 566–560 nm found by Bonner and Slater[14], who left open these two possible explanations of their experiments. However, antimycin does also induce a red shift in the spectrum, similar to that previously described for animal mitochondria[10,20,21], but the extent of which is much less[21,22]. The shift in plant mitochondria is only clearly demonstrable in the absence of the extra reduction that is simultaneously brought about by antimycin. Under the conditions of his experiment, Storey[19] would be unable to detect a red shift in the spectrum as this would be obscured by the much larger effect of antimycin on the redox state.

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