*Biochimica et Biophysica Acta*, 501 (1978) 440—448


BBA 47458

# THE EFFECTS OF UNCOUPLER ON THE RATES OF CYTOCHROME OXIDATION AND REDUCTION IN THE PHOTOSYNTHETIC BACTERIUM, *CHROMATIUM*

## EVIDENCE FOR A POSSIBLE CYTOCHROME SWITCHING

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(Received May 24th, 1977)
(Revised manuscript received September 2nd, 1977)

## Summary

The uncoupler, *m*-chlorocarbonyl cyanide phenylhydrazone (CCCP) added to anaerobic, dark-adapted whole cells of *Chromatium vinosum* is found to speed the reduction of cytochrome after oxidation by laser or by steady illumination and, subject to unknown factors, to slow the laser-induced oxidation. There is considerable evidence, including spectra and loss of low-temperature oxidizability that this results from a switch of the identity of the cytochrome oxidized from the low-potential cytochrome *c*-552 to the high-potential cytochrome *c*555. Redox control and/or control by conformational movements of the cytochromes, either being coupled to energy transduction in the cyclic system, are suggested as mechanisms for the switching. If the switching hypothesis is not accepted, the increased rate of reduction could alternatively be explained by postulating a phosphorylation site in the reduction pathway.

## Introduction

Investigations over the last few years on photosynthetic bacteria show a common feature that light induces both a cyclic and a non-cyclic electron transport [1—4]. The cyclic usually involves a high-potential *c*-type cytochrome and the non-cyclic a low-potential *c*-type cytochrome. Understanding of the

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Abbreviations: CCCP, *m*-chlorocarbonyl cyanide phenylhydrazone.

pathways for *Chromatium* is the result of a number of workers [5—9]. In *Chromatium* the low potential cytochrome *c*-552 (also called *c*-553 and *c*-423.5) oxidizes, following a flash of light, with a half-time of 1.0 μs [10—13]. Corresponding rates in other purple bacteria range from 0.3 to 80 μs [14—16,29]. Cytochrome *c*-552 reduces with half-times of 5—10 s following a flash or up to 5 min following a few minutes of steady illumination. Because of the slow reduction rates the steady-state level of oxidation of the low-potential cytochrome reaches near 100% values at rather low light intensities.

In *Chromatium* oxidation of the high-potential cytochrome *c*-555 (also called cytochrome *c*-422) is observed only when cytochrome *c*-552 is already oxidized. This condition can be brought about by steady illumination at levels sufficient to saturate the oxidation of low-potential cytochrome, or by raising the ambient redox potential with air, oxygen or other reagents. The half-time for cytochrome *c*-555 oxidation is 2.4 μs [11—13] and it reduces with a half-time of 0.1—0.01 s [11]. Rubin et al. [17] have measured and analyzed similar yet differing kinetics in the case of *Rhodopseudomonas* sp.

Phosphorylation by the electron transport systems has been studied by a number of workers. Very little is known about the location of the phosphorylation sites. Baltscheffsky [18] has found a crossover point between cytochrome $c_2$ and cytochrome *b* in *Rhodospirillum rubrum*, induced by ATP or pyrophosphate. This compares with the mitochondrial case in which a *b*-type cytochrome has been implicated in energy transduction [19]. These cytochromes are part of the cyclic electron transport system in *R. rubrum*. Cusanovich and Kamen [20] have associated phosphorylation in *Chromatium* with a redox potential sufficiently high (50—100 mV) to oxidize cytochrome *c*-552. It seemed to us that a study of the effect of uncouplers on the various cytochrome reaction rates should give some clues concerning phosphorylation sites. Morita et al. [7] report that CCCP markedly increases the rate of reduction of cytochrome *c*-552 in *Chromatium* whole cells. This note confirms their observation and reports further investigation of the effects of CCCP.

## Materials and Methods

We used *Chromatium vinosum* whole cells grown autotrophically in the medium described by Morita et al. [7]. The cells were harvested by centrifugation and resuspended in supernatant. The measurements of Fig. 1 were made on a dual-wavelength spectrophotometer designed by Chance [21] with sample activation by light from a Wolfram-Iodine incandescent lamp filtered with water and a Wratten 88A filter. The energy in the filtered band at the sample was about 10—15 mW/cm$^2$. In the experiments of Figs. 1, 2 and 4, the controls contained 10% ethanol to match whatever effect the ethanol used as solvent for the CCCP might have. The measurements of Figs. 2—4 were made with the single beam spectrophotometer using activation by a Q-switched ruby laser as described elsewhere [11,22,23] (pulses = ≈1 mJ/cm$^2$ at λ = 694 nm in 20—30 ns). The measuring light source for Fig. 4 was a 1000 W high-pressure mercury arc with Bausch and Lomb 250 mm grating monochromator. The measuring light source for Figs. 2 and 3 was a Wolfram-Iodine incandescent lamp with monochromator and was monitored with a beamsplitter and separate

photomultiplier whose signal was subtracted from the sample transmission signal after initial balancing.

## Results

Anaerobic cell suspensions showed normal cytochrome oxidation under steady illumination and reduction in the dark (Fig. 1, curve 1). The uppermost part of the curve, that is, the last oxidized and the first reduced has been interpreted [5,6] as cytochrome *c*-555 and the lower part, first oxidized and slowly reduced, as cytochrome *c*-552 in accordance with the characteristics of these cytochromes described in the introduction. Addition of CCCP (curves 2 and 3) caused a decrease in amplitude of light-induced cytochrome oxidation and either a speeding of its reduction or an elimination of the slow-recovery component.

Curve 3 at higher CCCP concentration shows partial restoration of slow recovery (although not as slow as the slow part of curve 1). This effect, surprising as it may be, was confirmed in later experiments designed to test the switching hypothesis discussed later. In these experiments Mr. Srinavasa Narayan, working under our direction, repeated the type of measurement shown in Fig. 1 many times at various measuring wavelength pairs in order to determine the spectra of the changes. His run at 0.12 mM CCCP showed curves like curve 2 except that an initial spike was even more prominent. The initial fast rise was largest at 555—558 nm (—540 nm reference) and recovered with a half-time of about 10 s to a steady level maintained until the activating light was turned off. The steady level before turn-off was highest at 551—553 nm, where it was about half of the initial peak. At 555—558 nm the steady level was nearly zero. The recovery half-time on turning the light off was 5 s or less.

His run at 0.30 mM CCCP gave curves shaped like curve 3 of Fig. 1. The

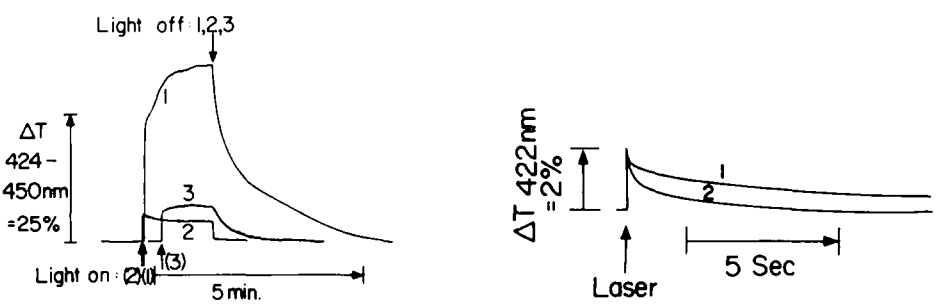



**Fig. 1. Effect of CCCP on photooxidation and dark reduction of cytochromes in *Chromatium*. Measurements made with dual-wavelength spectrophotometer (424—450 nm) and activation by steady illumination. Upward arrows indicate time of turning light on. Downward arrow, light off. Anaerobic, whole-cell suspensions. Bacteriochlorophyll concentration = 0.18 mM. Light path = 1.6 mm. $\Delta T$ = change in transmission as a fraction of the initial transmission. Curve 1, control (no CCCP); curve 2, with CCCP = 0.015 mM; curve 3, with CCCP = 0.15 mM.**

**Fig. 2. Effect of CCCP on reduction of laser-oxidized cytochrome in anaerobic whole cell suspension of *Chromatium*. Curve 1, control; curve 2, CCCP concentration = 0.15 mM. Bacteriochlorophyll concentration = 0.18 mM. Light path = 1.6 mm. Measuring $\lambda$ = 424 nm.**

recovery half-time after turning the light off was 20—30 s. The peak amplitude was at 555—560 nm.

When anaerobic cells are activated by single, short laser pulses, normally only the low potential cytochrome is oxidized [11]. The reduction then follows the time course shown in Fig. 2. Again, CCCP apparently speeds the reduction.

We sought next to investigate the effect on the high-potential cytochrome. We needed to choose a means for keeping the low-potential cytochrome oxidized so that the laser ould oxidize cytochrome *c*-555. Since we were using whole cells, poising the redox potential with redox mediators was not expected to be effective. Since CCCP had apparently speeded the reduction rate of what we thought was cytochrome *c*-552 we suspected that use of background illumination might fail to keep up with the reduction rate in the presence of CCCP. Therefore, we chose aeration.

The result was the series of measurements shown in Fig. 3. The anaerobic sample gave curve 1 which shows the slow reduction (not noticeable on this time scale) typical of the low potential cytochrome. The sample was then aerated and curve 2 was taken. About 70% of the cytochrome oxidized by the laser shows the 10 ms reduction half-time characteristic of cytochrome *c*-555. Curve 3 was taken after adding CCCP. and curve 4 after another aeration. Curve 4 seems inconsistent with curve 3. One might argue that the reduction rate of the low potential cytochrome has been speeded to the point where the aeration is insufficient to fully oxidize it. The laser, then, oxidized a larger proportion of low potential cytochrome, which, however, still appears slow on the time scale of Fig. 3. Because curve 3 is so much like curve 2 it is difficult to conclude that CCCP has any effect on the reduction rate of cytochrome *c*-555.

Because in the past, there have been suggestions of a phosphorylation site on or next to the reaction center bacteriochlorophyll, we looked at the effect

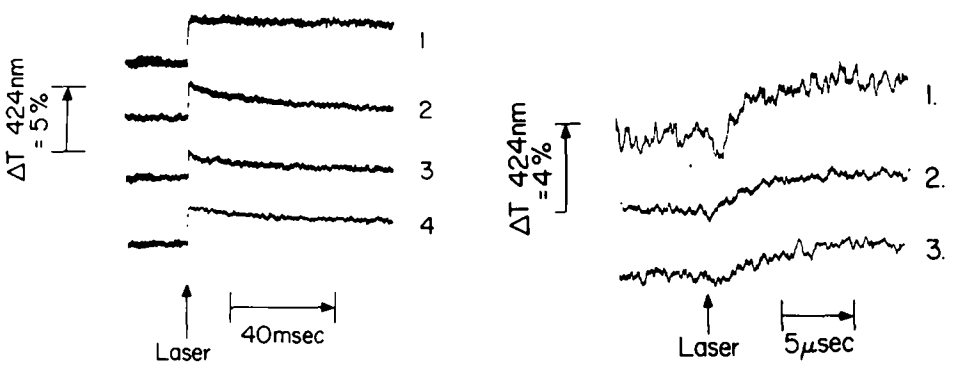



Fig. 3. Effect of aeration and CCCP on reduction of laser-oxidized cytochrome in *Chromatium* whole cells. Curve 1, anaerobic, no CCCP; curve 2, 5 min later, after aeration; curve 3, 8 min after curve 2, CCCP added to 0.15 mM; curve 4, 8 min after curve 3, after further aeration. Curves 3 and 4 were both followed by duplicate measurements, not reproduced here, but showing complete agreement. Bacteriochlorophyll concentration = 0.45 mM. Light path = 1.6 mm. Measuring λ = 424 nm.

Fig. 4. Effect of CCCP on laser-induced oxidation rates. Curve 1, same sample and cuvette as curve 1, Fig. 1; curve 2, same sample as curve 2, Fig. 1 (CCCP = 0.015 mM); curve 3, same sample as curve 3, Fig. 1 (CCCP = 0.15 mM). Measuring λ = 422.5 nm. Response time of detector set at 0.1 μs.

of CCCP on the rate of cytochrome oxidation. The result is shown in Fig. 4, done on anaerobic whole cells. Curve 1 shows the normal 1 $\mu$s half-time of oxidation following the dip due to the *P*-435 component. Curves 2 and 3 with increasing amounts of CCCP show a decreased amplitude and, surprisingly, an increase in half-time. Table I summarizes measurements of the oxidation rate. Fig. 4 presents three of the five photographs of Series "19.5.71". Only one control picture was taken because the half-time under such conditions is well known [24]. The oscilloscope photographs were measured by fitting visually to electronically generated exponential curves. In series 19.5.71 each photograph was so measured several times to test the reproducibility of the method of measuring.

The second series of Table I agrees with the first that the cytochrome oxidation is slower with CCCP present. The third series disagrees. We have not found the cause of this variation. We suspected the CCCP solution and made up a new solution which gave the same results (all included in the seven photographs of Expt. 2 which were made with various concentrations of CCCP within the range given). A spectrum of the laser-induced changes in the 550—560 nm region made the day before the series 17.12.71 also showed very little influence of CCCP (unlike Fig. 5). We conclude from Table I that a phenomenon showing increased oxidation halftime in the presence of CCCP exists, but all the factors influencing it are not yet known.

After the series 19.5.71 (and before the others) it occurred to us that the effects seen in Figs. 1, 2 and 4 are consistent with a hypothesis that the uncoupler caused a switch-over from cytochrome *c*-552 to cytochrome *c*-555 oxidation in the anaerobic samples. This was tested by measuring the spectra and the low-temperature dependence of the cytochrome oxidation.

The laser-induced spectrum, in the $\alpha$-region, is shown in Fig. 5. The data come from the same set of experiments as those labelled "series 8.11.71" in Table I. The two spectra are very similar to those obtained with and without background illumination (see Figs. 4 and 6 of ref. 9 as example), the relative amplitudes at different wavelengths being more diagnostic than the position of the peaks. It appears that the cytochrome oxidized by the laser in the presence of CCCP is cytochrome *c*-555. The failures to obtain similar evidence of switching to cytochrome *c*-555 in connection with the series designated

TABLE I

RATE OF LASER-INDUCED CYTOCHROME OXIDATION

| Series | Expt. | CCCP concn. (mM) | No. of pictures | No. of measurements of pictures | $t_{1/2}$ · S.E. ($\mu$s) |
|---|---|---|---|---|---|
| 19-5-71 | 1 | 0 | 1 | 6 | 1.29 ± 0.04 |
| | 2 | 0.015 | 2 | 5 | 1.82 ± 0.03 |
| | 3 | 0.15 | 2 | 6 | 2.45 ± 0.22 |
| 8-11-71 | 1 | 0 | 5 | 5 | 1.01 ± 0.04 |
| | 2 | 0.06 | 2 | 2 * | 1.4 ± 0.2 * |
| 17-12-71 | 1 | 0 | 5 | 5 | 1.19 ± 0.08 |
| | 2 | 0.1—0.3 | 7 | 7 | 1.14 ± 0.10 |

* Standard error estimated from measurements of acceptable upper and lower limits.

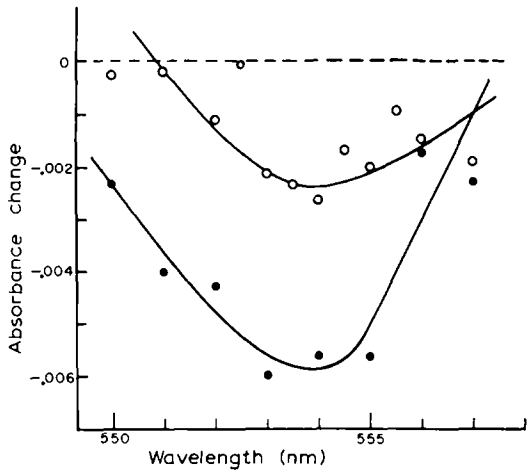



Fig. 5. Effect of CCCP on laser-induced difference spectrum of cytochrome oxidation. Anaerobic whole cell suspension of *Chromatium*. •, control, no CCCP; ○, with CCCP = 0.06 mM. Both curves: Bacteriochlorophyll concentration = 0.27 mM. Path length = 1.6 mm. Slit-width of monochromator corresponded to 1.7 nm of wavelength. Data obtained by Mr. Srinavasa Narayan. The peak positions of the two spectra are difficult to distinguish but the difference in amplitude from 550 to 554 nm and the similarity in amplitude around 556—557 nm distinguishes between the two cytochromes as shown in Figs. 4 and 6 of ref. 9.

17.12.71 in Table I was mentioned above. The spectra induced by steady illumination were already described in connection with Fig. 1. It may be noted that the latter appear to give better-resolved peaks than the laser-induced spectra. They indicate that at lower CCCP concentration the initial cytochrome oxidized is cytochrome *c*-555 but that this changes to cytochrome *c*-552 in the steady state formed after a few seconds, while at higher concentrations of uncoupler the cytochrome oxidized is cytochrome *c*-555 only. An attempt was made to determine the spectral changes produced by adding uncoupler with no illumination but we did not succeed in getting interpretable data.

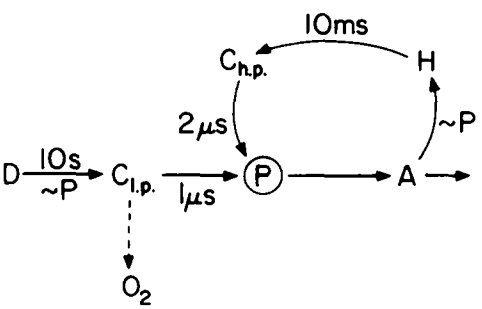
Cytochrome *c*-552 is well known to be oxidized by light at liquid nitrogen temperatures [25] while cytochrome *c*-555 is not. Mr. Anthony Mishik, under our direction, froze samples of *Chromatium* whole cells with and without CCCP in the dark with liquid nitrogen. Monitored in a dual-wavelength spectrometer the samples without CCCP showed irreversible absorbance decrease at 423—450 nm upon illumination while that with CCCP showed only a smaller, reversible absorbance increase at 423—450 nm. These are interpreted as cytochrome oxidation without CCCP and none with CCCP (only *P*-435, oxidized bacteriochlorophyll).

## Discussion

We will offer three different suggestions to explain these data. The first suggestion ignores the evidence for the switching hypothesis and simply notes that reduction of presumed cytochrome *c*-552 was speeded by uncoupler, and that this could indicate a phosphorylation site between cytochrome *c*-552 and the external electron donor which is presumed to be the source of reducing power for this cytochrome. Probably everyone agrees that there is a phos-

phorylation site in the cyclic system. However, our experiments found no evidence that uncoupler speeds the electron transport in this system. A tentative explanation can be made by placing a hypothetical "buffer" pool of electron carriers between the high-potential cytochrome and the phosphorylation site. Since this carrier needs to have a high redox midpoint potential we repeat a previous suggestion [26] that this component might be the high-potential iron protein (HiPIP) isolated by Bartsch [27]. This suggestion is summarized in Fig. 6.

Our second suggestion accepts the evidence that the uncoupler causes a switch-over from cytochrome *c*-552 oxidation to cytochrome *c*-555 oxidation by light under anaerobic conditions. This evidence may be summarized by noting that CCCP causes: (a) slower oxidation rate (except in series 17.12.71 of Table I), (b) faster reduction rate in the dark, possibly slowing somewhat at higher concentration, (c) smaller amplitude of absorbance changes, similar to effects of using background illumination to induce cytochrome *c*-555 oxidation by holding cytochrome *c*-552 oxidized, (d) spectra corresponding to cytochrome *c*-555 with both steady illumination and laser and (e) loss of cytochrome oxidizibility at 77°K. A possible explanation of such a phenomenon is summarized in Fig. 7. The phosphorylation site is shown as a "black box" which can supposedly be either a chemical intermediate type or a chemiosmotic loop. It is postulated that the high potential end of the box is in redox communication with cytochrome *c*-555 and that the low potential end approaches redox equilibrium with cytochrome *c*-552. (To avoid an electrical short-circuit of the box one assumes that the redox potentials, $E_h$, of the various components can differ from each other and are prevented by reaction



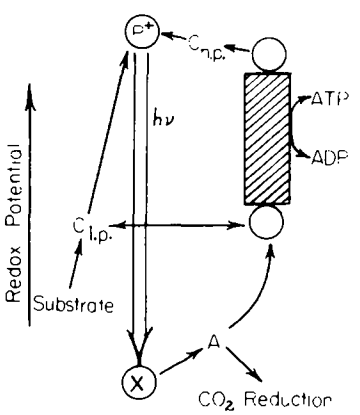




Fig. 6. Scheme summarizing suggestion 1. Arrow indicate electron movements. A = electron acceptor at the reaction center. P = reaction center bacteriochlorophyll. $C_{l.p.}$ = cytochrome *c*-552. $C_{h.p.}$ = cytochrome *c*-555. D = substrate electron donor(s). H = electron carrier pool (HiPIP?) which buffers effect of uncoupler on reduction rate of cytochrome *c*-555. ~P = phosphorylation site.

Fig. 7. Scheme summarizing suggestion 2. Arrows (except those between ATP and ADP) show electron movements, P+ represents reaction center bacteriochlorophyll oxidized by light absorption. X = primary electron acceptor. A = secondary acceptor. $C_{l.p.}$ = cytochrome *c*-552. $C_{h.p.}$ = cytochrome *c*-555. Cytochrome *c*-552 is postulated to equilibrate with the low-potential side of the energy transduction site "black box" of the cyclic electron transport system and thus to go oxidized when the ATP/ADP ratio is small. This would shut off the substrate-to-$CO_2$ electron flow and start up the cyclic system.

specificity or by locality from communicating with other components except those specifically designated for given functions [28]. We are not proposing or discussing any variation of midpoint potentials ($E_M$ or $E^0$) such as might be found inside the "black box".) Uncoupler would cause collapse of the potential difference across the site. This could result in a lowering of the potential ($E_h$) at cytochrome *c*-555 or a raising of the potential at cytochrome *c*-552 or both. We assume further that some redox reservoir stabilizes the potential at cytochrome *c*-555 enough so that the redox potential "seen" by cytochrome *c*-552 is raised and cytochrome *c*-552 thus becomes oxidized. Illumination will then produce cytochrome *c*-555 oxidation.

The function of such a switching system can be seen as follows: When the ATP concentration is high (in the dark this could be produced by glycolytic phosphorylation and low demand) cytochrome *c*-555 is reduced and the first photons received will induce electron transport from substrate through cytochrome *c*-552 to $CO_2$ fixation. The $CO_2$ fixation will demand ATP and as the ATP/ADP ratio decreases cytochrome *c*-552 will go oxidized and the next photons received will cause cytochrome *c*-552 oxidation and cyclic phosphorylation. When the ATP demand has been met the resulting increase of potential across the phosphorylation site will reduce cytochrome *c*-552 and allow more electron transport from substrate. A perfect balance between the two electron transport systems would result. The finding of Cusanovich and Kamen [20], mentioned in the introduction, that photo-phosphorylation in *Chromatium* proceeds best at a potential which would oxidize cytochrome *c*-552 fits this suggestion well.

Our third suggestion is that some kind of movement of the cytochromes with respect to the reaction center may be involved in the switching [29]. The oxidation half-times of the two cytochromes are near enough to each other (1.0 $\mu$s vs. 2.0—2.4 $\mu$s) that if these rates alone controlled then when both cytochromes are initially in the reduced state a laser pulse should cause some oxidation of cytochrome *c*-555. We know of no evidence that this happens. It may, therefore, be that cytochrome *c*-555 has an unfavorable position with respect to the reaction center when the ATP is high and that collapse of the ATP/ADP ratio, as with uncoupler, causes cytochrome *c*-555 to move to a more favorable position. This could fit in with suggestions that conformational changes play a role in phosphorylation.

## Acknowledgements

The authors wish to thank the International Research and Exchanges Board for making this brief collaboration possible and the National Science Foundation Grants BMS 71-00765, PCM 76-15724 and PCM76-23744 for financial support. We are also grateful for the help of Messrs. Robert Burgess, Srinavasa Narayan, and Anthony Mishik.

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