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CONVERSION OF BIOMEMBRANE-PRODUCED ENERGY INTO ELECTRIC FORM

III. CHROMATOPHORES OF *RHODOSPIRILLUM RUBRUM*

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SUMMARY

The mechanisms of energy coupling and ion transport in *Rhodospirillum rubrum* chromatophores have been studied. Photoreduction of NAD^+ and photophosphorylation have been measured under anaerobic conditions in the presence of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), ascorbate and antimycin A. The uncoupler *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone (FCCP), as well as ADP+phosphate, has been found to inhibit the photoreduction of NAD^+ in this system. Addition of an electron acceptor, such as methylviologen, fumarate or O_2, to antimycin-treated chromatophores initiates the process of photophosphorylation.

Chromatophores of *R. rubrum* accumulate the penetrating anions, phenyl dicarboundecaborane and tetraphenyl boron, as well as iodide, if the I^- carrier, di-(pentafluorophenyl) mercury, is added. The anion accumulation can be supported by light-induced cyclic electron flow ($NADH \rightarrow O_2$, succinate$\rightarrow$ ferricyanide), by hydrolysis of ATP or inorganic pyrophosphate, as well as by reversal of the energy-requiring transhydrogenase reaction ($NADPH \rightarrow NAD^+$). The type of energy source influences only the extent of the anion accumulation process.

Cessation of the energy supply (*e.g.* by exhaustion of the energy source or poisoning of the system by specific inhibitors or an uncoupler) brings about an efflux of the accumulated anions. Uptake of anions is accompanied by alkalinization of the outer solution; release of anions is accompanied by acidification.

It is concluded that there is an energy-dependent charge-specific mechanism for anion accumulation in the chromatophore membrane resembling that found in the membrane of submitochondrial particles. It is stated that the electric field (the "*plus*" inside the chromatophore) is the motive force for ion transfer through the chromatophore membrane against a concentration gradient. The data on NAD^+ photoreduction, noncyclic photophosphorylation and energy-dependent anion transport are summarized as the concept of four sites of energy coupling in the chromato-

Abbreviations: PCB^-, phenyl dicarboundecaborane anion; DDA^+, *N,N*-dibenzyl *N,N*-dimethyl ammonium cation; FCCP, *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

phore redox chain localized at the same steps as in animal mitochondria (NADPH → NAD^+, NADH → cytochrome *b*, cytochrome *b* → cytochrome *c*, and the region after cytochrome *c*). Each of these coupling sites can provide energy for generation of a membrane potential.

INTRODUCTION

The data summarized in the two previous papers[1,2] suggest the existence of a mechanism for ion accumulation localized in the mitochondrial membrane, which is specific only for the sign of charge of a penetrating ion. The directions of movement of cations and anions carried by this mechanism are opposite. It was pointed out that the charge-specific transport of penetrating ions across the membrane of mitochondria and submitochondrial particles is driven by an electric field orientated across the membrane and supported by respiration or ATP hydrolysis. In this paper the same approach has been applied to the study of chromatophores of the photosynthetic bacterium *Rhodospirillum rubrum*.

METHODS

Preparation of chromatophores

The purple bacterium *R. rubrum* was grown for 3–4 days under anaerobic conditions in a medium containing sodium succinate or malate and yeast autolysate in a luminostat (about 1000 luxes) at 30° (ref. 3). Bacterial cells were sedimented at 1200 × *g* for 30 min. Subsequent operations were carried out at 0–2°. The cells washed twice in a medium containing 0.25 M sucrose and 0.05 M Tris–HCl (pH 7.6) were used for the preparation of chromatophores. The latter procedure was similar to that used for the preparation of submitochondrial particles[1]. Bacterial cells were disrupted in an MSE 500-W ultrasonic disintegrator at a frequency of 20 kcycles for 3 min in a solution containing 0.25 M sucrose, 0.005 M $MgCl_2$, 0.05 M Tris–HCl (pH 7.6). The mixture was centrifuged at 40000 × *g* for 15 min to separate the undisrupted cells and cell debris. The sediment was removed and the supernatant centrifuged again at 150000 × *g* for 1 h. The sedimented chromatophores were suspended in a solution of the composition given above kept in a Thunberg tube under argon at 0°. Energy-linked processes were initiated by the light of a tungsten lamp passing through an F-880 interference filter. The light intensity was $1 \cdot 10^{-9}$ Einstein $\cdot cm^{-2} \cdot sec^{-1}$.

The concentration of bacteriochlorophyll was determined spectrophotometrically. The coefficient of molar extinction used was 140 $mM^{-1} \cdot cm^{-1}$ at 880 nm. In the anaerobic experiments a Thunberg cuvette filled with argon was used.

For other methods see ref. 1.

RESULTS AND DISCUSSION

Localization of the energy-coupling sites in the redox chain of chromatophores

It has been shown that the cyclic photosynthetic electron transport chain, including bacteriochlorophyll, ubiquinone and cytochromes of *b* and *c* types, is coupled to phosphorylation at two points, one localized between cytochromes *b* and *c* and the other between bacteriochlorophyll and cytochrome *b* or between cytochrome *c* and

bacteriochlorophyll (for review see ref. 4). It has been proposed[5–11] that an additional coupling site is localized between NAD^+ and cytochrome *b*, being associated with the system for NAD^+ photoreduction. In fact, photoreduction of NAD^+ in chromatophores resembles reversed electron transfer *via* the first coupling site in the mitochondrial redox chain. NAD^+ reduction can be driven by ATP hydrolysis. Reduction of NAD^+ supported by light or ATP is inhibited by uncouplers, energy acceptors and rotenone[6,8,9].

The alternative possibility is that NAD^+ photoreduction proceeds as a direct energy-independent electron transfer like the photoreduction of $NADP^+$ in green plants[12]. Dark ATP-driven NAD^+ reduction, whose rate is much lower than that in the light, would be accomplished by a special electron transfer system different from that participating in NAD^+ photoreduction.

The sensitivity of NAD^+ photoreduction to rotenone is not decisive for identifying the energy-coupling site since some energy-independent dehydrogenases were also found to be inhibited by rotenone[13,14].

Inhibition of NAD^+ photoreduction by uncouplers and substrates for phosphorylation would have been considered as the final evidence for reversed electron transfer but for the possibility mentioned by JONES AND VERNON[9]. As they have noted, "if cyclic electron flow was so tightly coupled to phosphorylation, such that by the addition of an uncoupler (or of an active phosphorylation system) cyclic flow became considerably stimulated, then this could cause a draining away of electrons from the direct pathway of NAD^+ reduction". In order to exclude this possibility the sensitivity of NAD^+ photoreduction to uncouplers was tested under conditions preventing the operation of alternative electron transfer pathways. Chromatophores were incubated in the presence of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD)[1], ascorbate and NAD^+, in an anaerobic cuvette. Upon illumination, NAD^+ was reduced (Fig. 1, Curve 1), the rate of reduction being somewhat lower when the mixture was supplemented with antimycin (Curve 2). Addition of the uncoupler *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone (FCCP), at a concentration of $1 \cdot 10^{-6}$ M, to antimycin-treated chromatophores completely inhibited photoreduction of NAD^+ (Curve 3). The effect of the uncoupler in the experiment described above cannot be explained by the stimulation of competitive cyclic electron transfer which was interrupted by antimycin. Respiratory pathways could not operate because of the absence of oxygen. The possibility of shunting the antimycin site by TMPD present in the system[5,15] was ruled out by the experiment shown in Fig. 2 in which the kinetics of photophosphorylation under anaerobic conditions was measured. It is seen that antimycin inhibits photophosphorylation in spite of the presence of TMPD (*cf.* Curves 1 and 3). Apparently TMPD is completely reduced by ascorbate and hence cannot operate as an electron acceptor.

Under the same conditions photoreduction of NAD^+ was inhibited by ADP and phosphate[16].

It should be mentioned that uncouplers and energy acceptors do not inhibit but stimulate $NADP^+$ photoreduction in green plants[17].

The above data show unambiguously that photoreduction of NAD^+ in *R. rubrum* chromatophores occurs *via* an energy-dependent reversed electron transfer pathway. This means that there is an energy-coupling site in the NADH-dehydrogenase segment of the chromatophore redox chain.

The data presented in Fig. 2 also suggest that the antimycin-insensitive coupling site of electron transfer operating upon addition of methylviologen, O_2 or fumarate, is localized between a cytochrome of *c* type and the "bacteriochlorophyll–primary electron acceptor" system. This region is the only step in the electron transfer system capable of producing utilizable energy in the chain "ascorbate→ TMPD→ cytochrome *c*→ bacteriochlorophyll→ methylviologen" (see ref. 16).

Fig. 1. The effect of antimycin A and FCCP on NAD^+ photoreduction by *R. rubrum* chromatophores under anaerobic conditions. Incubation mixture: 0.25 M sucrose; 0.05 M Tris buffer (pH 7.8); $5 \cdot 10^{-3}$ M $MgCl_2$; $1 \cdot 10^{-3}$ M NAD^+; $1 \cdot 10^{-4}$ M TMPD; $1 \cdot 10^{-3}$ M ascorbate; 1 mg/ml of bovine serum albumin and chromatophores, 25 μg of bacteriochlorophyll/ml. 1, control; 2, with $1 \cdot 10^{-6}$ M antimycin; 3, with antimycin and $1 \cdot 10^{-6}$ M FCCP.

Fig. 2. Photophosphorylation in chromatophores of *R. rubrum*. The rate of phosphorylation was measured fluorimetrically using the system "hexokinase + glucose + glucose-6-phosphate dehydrogenase + $NADP^+$" (for details see ref. 16). Incubation mixture: 0.25 M sucrose; 0.05 M Tris buffer (pH 7.6); $5 \cdot 10^{-3}$ M $MgCl_2$; $5 \cdot 10^{-3}$ M potassium phosphate; $2 \cdot 10^{-4}$ M ADP; $1 \cdot 10^{-3}$ M AMP; 0.02 M glucose; $1 \cdot 10^{-3}$ M $NADP^+$; hexokinase, 0.4 mg/ml; glucose-6-phosphate dehydrogenase, 10 μg; chromatophores, 25 μg of bacteriochlorophyll/ml. Anaerobic conditions. 1, without additions; 2, $1 \cdot 10^{-4}$ M TMPD; $1 \cdot 10^{-3}$ M ascorbate; $1 \cdot 10^{-6}$ M antimycin A; $1 \cdot 10^{-3}$ M methylviologen. 3, TMPD; ascorbate; antimycin. 4, TMPD; ascorbate; antimycin; $1 \cdot 10^{-3}$ M fumarate. 5, TMPD; ascorbate; antimycin; methylviologen; $1 \cdot 10^{-6}$ M FCCP.

Thus, it can be assumed that the redox chain of *R. rubrum* chromatophores includes energy-coupling sites localized on the same levels as those of the respiratory chain of animal mitochondria, namely (1) in the NADH-dehydrogenase region, (2) between cytochromes *b* and *c*, and (3) after cytochrome *c*. The first coupling site of the chromatophore redox chain is engaged in NAD^+ photoreduction, the second and the third being responsible for photophosphorylation. One more coupling site in both mitochondria and chromatophores might be associated with the transhydrogenase reaction.

Energy-linked transport of anions penetrating into chromatophores

The transport of anions penetrating into chromatophores was measured as described in the first paper of this series[1]. Fig. 3 shows the effect of chromatophores on the concentration of the penetrating anion, phenyl dicarbaundecaborane (PCB^-), in solution. It is seen that addition of chromatophores in the dark brings about a decrease in PCB^- concentration. This process corresponds to the passive absorption of PCB^- anions shown earlier for phospholipid micelles, mitochondria and submitochondrial particles[1,2].

Illumination induces additional uptake of PCB^- anions. Switching off the light source is followed by release of the anions that had accumulated in the light. The

same responses can be observed if PCB^- is substituted by another penetrating anion, tetraphenyl boron (similar results were also obtained with picrate). In Fig. 3, an experiment with the iodide anion is also shown. I^- has low affinity for lipids. It penetrates membranes easily only in the presence of a carrier. Di(pentafluorophenyl) mercury was found to be an effective iodide carrier. Fig. 3 shows that I^- anions, added to chromatophores in the presence of di(pentafluorophenyl) mercury, are taken up under illumination and released after switching off the light, as was observed with the anions PCB^- and tetraphenyl boron. Unlike these two penetrating anions, however, iodide is not passively absorbed by chromatophores in the dark. This result clearly shows that the anion response to the transition from a deenergized to an energized state does not require a prephase of passive absorption of anions by the chromatophores. This response can be demonstrated with ions which are not bound by membranes.

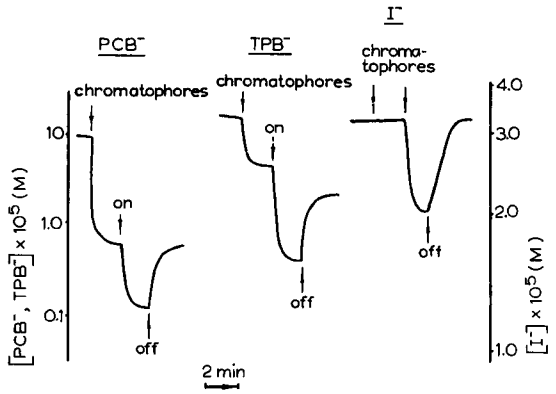

Fig. 3. Accumulation of PCB^-, tetraphenyl boron (TPB^-) and I^- anions by chromatophores of *R. rubrum*. Incubation mixture: 0.25 M sucrose, 0.03 M Tris buffer (pH 7.5), $5 \cdot 10^{-3}$ M $MgSO_4$, chromatophores (0.09 mg of bacteriochlorophyll/ml). In the sample with I^- $1 \cdot 10^{-5}$ M di(pentafluorophenyl) mercury was added.

The effect of antimycin and TMPD on light-induced uptake of PCB^- anions is shown in Fig. 4. It is seen that inhibition of the cyclic electron transport chain by antimycin results in an efflux of the PCB^- that had accumulated during illumination.

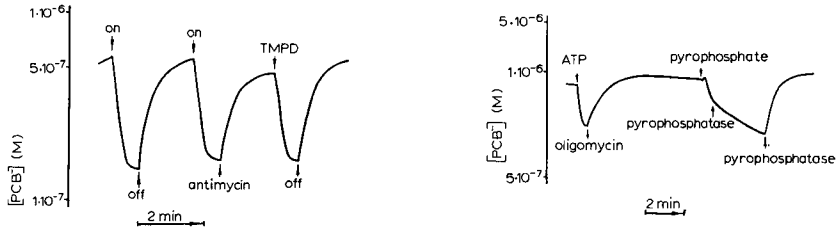

Fig. 4. The effect of antimycin and TMPD on the photoinduced uptake of PCB^- anions by chromatophores of *R. rubrum*. Incubation mixture: 0.25 M sucrose, 0.05 M Tris buffer (pH 7.5), chromatophores (0.012 mg of bacteriochlorophyll/ml). Additions: $2 \cdot 10^{-6}$ M antimycin, $4 \cdot 10^{-5}$ M TMPD.

Fig. 5. PCB^- anion accumulation in the presence of ATP or inorganic pyrophosphate. Conditions as in Fig. 4. Additions: $1 \cdot 10^{-3}$ M ATP, oligomycin (2.6 μg/ml), $1 \cdot 10^{-3}$ M inorganic pyrophosphate, yeast pyrophosphatase (0.02 mg/ml). Activity about 5 mg of pyrophosphate/mg of protein per min.

Shunting of the antimycin-sensitive point with TMPD restores the light-dependent uptake of PCB^- anions. In the latter case, energy is supplied by the operation of a coupling site localized between cytochrome *c* and the "bacteriochlorophyll–primary electron acceptor(s)" system.

Fig. 5 shows the effect of ATP and inorganic pyrophosphate. It is seen that anion accumulation in the dark can be supported by ATP, the process being sensitive to oligomycin. Addition of inorganic pyrophosphate after oligomycin reinitiates anion uptake. Removal of pyrophosphate by added pyrophosphatase leads to a loss of the accumulated PCB^-.

Fig. 6 illustrates PCB^- accumulation at the expense of the energy generated in the first coupling site of the chromatophore redox chain. After the light–dark cycle, which is demonstrated here for comparison, addition of NADH induces the uptake of the PCB^- anions. Under these (aerobic) conditions, NADH is oxidized by oxygen *via*

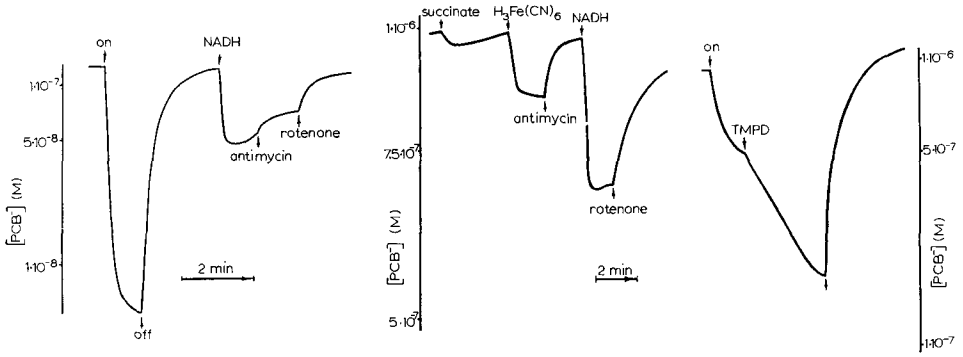

Fig. 6. PCB^- anion accumulation using light energy or at the expense of NADH oxidation in the dark. Incubation mixture (as for Fig. 4) contained chromatophores, 0.02 mg of bacteriochlorophyll/ml. Additions: $1 \cdot 10^{-3}$ M NADH, $3 \cdot 10^{-6}$ M antimycin, $2 \cdot 10^{-6}$ M rotenone.

Fig. 7. PCB^- anion accumulation supported by electron transfer in the dark *via* different coupling sites of the chromatophore redox chain. Conditions as in Fig. 4. Additions: 0.01 M succinate, $4 \cdot 10^{-3}$ M $K_3Fe(CN)_6$, $4 \cdot 10^{-6}$ M antimycin, $2 \cdot 10^{-6}$ M rotenone, $2 \cdot 10^{-4}$ M TMPD, $1 \cdot 10^{-3}$ M NADH.

the antimycin-insensitive pathway. Addition of $3 \cdot 10^{-6}$ M antimycin has little effect on the PCB^- level, whereas rotenone induces the efflux of all the PCB^- anions accumulated during NADH oxidation.

Accumulation of PCB^- anions supported by electron transfer in the dark *via* different coupling sites of the redox chain is demonstrated in Fig. 7. It is seen that addition of ferricyanide to chromatophores treated with succinate results in the uptake of PCB^-. In this case, electron transfer *via* the second site of energy coupling (cytochrome *b*–cytochrome *c* region) is operative. Addition of antimycin induces the efflux of PCB^- anions. Subsequent addition of NADH initiates PCB^- accumulation supported by electron transfer *via* the first coupling site. Rotenone abolishes the effect of NADH. Addition of TMPD with illumination again induces PCB^- uptake. In the last case, the third coupling site is responsible for the production of utilizable energy.

Addition of NADPH and NAD^+ to chromatophores in the dark initiates the accumulation of PCB^- anions (Fig. 8A). In this case, the energy for active transport is provided by reversal of the energy-requiring transhydrogenase reaction. This phe-

nomenon is similar to that described for submitochondrial particles[1]. Subsequent additions of NADH and $NADP^+$, resulting in the equalization of the concentrations of NADPH, $NADP^+$, NADH and NAD^+, induce the loss of the whole of the accumulated PCB^-.

Another experiment demonstrating transhydrogenase-dependent anion accumulation is shown in Fig. 8B. The conditions were somewhat different from those used in Fig. 8A; the process was carried out in the light, cyclic electron flow being interrupted by antimycin. The reaction mixture included lactate dehydrogenase and rotenone. It can be seen that the addition of NADPH has little effect; subsequent addition of NAD^+ initiates the active uptake of PCB^-. The process is partially reversed by addition of the reaction product, NADH. This effect is abolished by pyruvate oxidizing NADH. Addition of $NADP^+$, another substrate for the energy-linked transhydrogenase, induces PCB^- efflux.

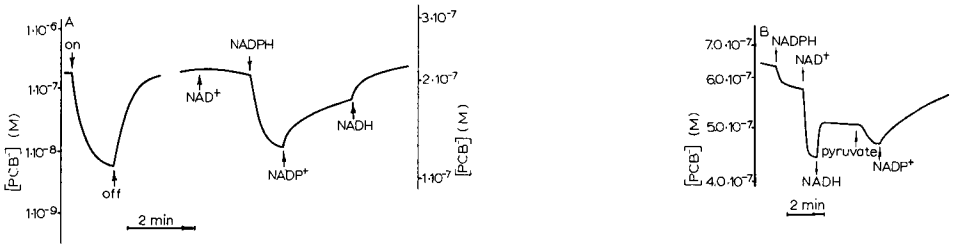

Fig. 8. PCB^- anion accumulation induced by reversal of the energy-requiring transhydrogenase reaction. Incubation mixture: 0.25 M sucrose, 0.05 M Tris buffer (pH 7.5), $2 \cdot 10^{-6}$ M rotenone, chromatophores (0.024 mg of bacteriochlorophyll/ml). Additions: A. $1 \cdot 10^{-3}$ M NAD^+, NADPH, NADH, $NADP^+$. B. $1 \cdot 10^{-3}$ M NADPH, $2 \cdot 10^{-3}$ M NAD^+, $1 \cdot 10^{-3}$ M NADH, $2 \cdot 10^{-3}$ M pyruvate, $2 \cdot 10^{-3}$ M $NADP^+$. The incubation mixture was supplemented with lactate dehydrogenase (0.02 mg/ml).

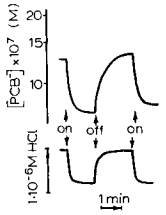

Fig. 9. pH changes accompanying energy-dependent PCB^- uptake by chromatophores of *R. rubrum*. Incubation mixture: 0.25 M sucrose, $5 \cdot 10^{-3}$ M Tris buffer (pH 7.5), 0.025 M KCl, $5 \cdot 10^{-3}$ M $MgSO_4$.

The results of simultaneous measurements of PCB^- concentration and pH are presented in Fig. 9. It is seen that accumulation of PCB^- anions in the light is accompanied by the uptake of an equal amount of H^+. Release of PCB^- after switching off the light is accompanied by an efflux of the same amount of H^+ into the medium. This result confirms the conclusion made previously in regard to mitochondria and submitochondrial particles[1,2], that the transport of penetrating ions is associated with the work of a proton pump.

In other experiments, it was shown that accumulation of penetrating anions inhibits other energy-linked functions of chromatophores, *e.g.* reduction of NAD^+ by

ascorbate and the energy-requiring transhydrogenase reaction. Under the same conditions the penetrating cation, *N*,*N*-dibenzyl *N*,*N*-dimethyl ammonium (DDA^+), was ineffective.

JACKSON *et al.*[18] reported recently that illuminated valinomycin-treated chromatophores do not accumulate K^+ but rather extrude it. Switching off the light resulted in an influx of K^+. Thus, the flows of anions and cations arising on the transition of chromatophores into an energized state have opposite directions: anions move into, and cations move out of, the chromatophores.

This result is unequivocal evidence in favor of the original suggestion of MITCHELL[19,20], that in the energized state there exists an electric potential difference on the chromatophore membrane, the "*plus*" sign being inside.

It is noteworthy that the use of the accumulation of penetrating anions as a probe for the energized state independently confirms the localization of coupling sites which was suggested on the basis of the study of NAD^+ photoreduction and photophosphorylation in chromatophores of *R. rubrum*. These sites are: the NADH-dehydrogenase region; cytochromes *b*–*c* step, and the region after cytochrome *c*. The penetrating anion test allows one to demonstrate one more site of energy production associated with the reversal of the energy-requiring transhydrogenase reaction.

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