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THE ROLE OF SOLUBLE CYTOCHROME *c*-551 IN CYCLIC ELECTRON FLOW-DRIVEN ACTIVE TRANSPORT IN *CHROMATIUM VINOSUM*

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Summary

Spheroplasts have been prepared from the photosynthetic purple sulfur bacterium *Chromatium vinosum* by lysozyme plus ethylenediaminetetraacetic acid treatment. These spheroplasts are able to take up alanine in the light, but light-dependent alanine uptake is lost upon subsequent washing of the spheroplasts. The observations that alanine uptake driven by a potassium plus valinomycin-induced membrane potential (outside positive) is not affected by washing and that light-dependent alanine uptake can be restored by addition of the supernatant from washing suggest that a soluble electron carrier is lost during washing. Light-dependent alanine uptake in washed spheroplasts could be restored by addition of *C. vinosum* cytochrome *c*-551. Other soluble electron carriers from *C. vinosum* (high-potential iron protein, cytochrome '*f*', cytochrome *c'* and the flavocytochrome *c*-552) did not restore alanine uptake nor did a variety of other soluble electron carrier proteins from other organisms. These results suggest that cytochrome *c*-551 functions as an electron carrier in the cyclic electron transfer chain of *C. vinosum*. Mitochondrial cytochrome *c* (equine heart) and cytochrome *c*-551 from *Pseudomonas aeruginosa* were highly effective in restoring light-dependent alanine uptake in washed spheroplasts, making it likely that *C. vinosum* cytochrome *c*-551 is related by evolution to the same cytochrome *c* family as these other two *c* cytochromes.

Abbreviations: HOQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; EDTA, ethylenediaminetetraacetic acid; E_m, midpoint oxidation-reduction potential; DCCD, *N,N'*-dicyclohexylcarbodiimide; Hipip, high-potential iron protein; $\Delta\psi$, membrane potential; $\Delta\mu_{H^+}$, electrochemical proton gradient.

Introduction

It has been known for some time that *Chromatium vinosum* contains two membrane-bound *c*-type cytochromes, low-potential (E_m = +10 mV) cytochrome *c*-553 and high-potential (E_m = +340 mV) cytochrome *c*-555 [1]. Both cytochromes [2—5] can serve as efficient, direct electron donors to the photooxidized reaction center bacteriochlorophyll (P-870$^+$). Although cytochrome *c*-553 is oxidized preferentially when both cytochromes are reduced, the reduction of oxidized cytochrome *c*-553 after a flash is so slow that it appears unlikely that cytochrome *c*-553 makes a significant contribution to cyclic electron flow in *C. vinosum* in vivo.

Although cytochrome *c*-555 has been widely assumed to be the functional electron donor to P-870$^+$ during electron flow in *C. vinosum*, kinetic measurements of cytochrome *c*-555 in cell-free preparations (chromatophores) indicated that the reduction of oxidized cytochrome *c*-555 after a flash was considerably slower than expected for a participant in a cyclic pathway [2—5]. Recently, van Grondelle et al. [6] have provided evidence from flash spectroscopy that the electron donor to oxidized cytochrome *c*-555 in *C. vinosum* is a soluble *c* cytochrome (cytochrome *c*-551), most of which is lost during chromatophore preparation. We have obtained direct evidence from reconstitution studies (using light-dependent alanine uptake by *C. vinosum* [7] as a measure of cyclic electron flow) to support this hypothesis. We have also obtained evidence that *C. vinosum* cytochrome *c*-551 probably belongs to the same family of *c*-type cytochromes as do mitochondrial cytochrome *c* and *Pseudomonas aeruginosa* cytochrome *c*-551.

Methods

C. vinosum was grown on the malate-containing medium described previously [7]. Spheroplasts were prepared by a modification of the method of Miura and Muzushima [8]. The cells were washed once in cold distilled water and resuspended in 25 mM Tris-HCl (pH 8.0), 0.45 M sucrose and 1.3 mM EDTA (1 g wet weight of cells/80 ml). 25 mg of lysozyme/80 ml were added and the cells incubated for 60 min at 31—32° C. The spheroplasts were collected by centrifugation (5000 × g, 15 min) and resuspended in 0.5 M sucrose, 5 mM $MgCl_2$, 5 mM sodium phosphate (pH 7.4). Washed spherplasts were prepared by washing in the resuspension buffer, to which KCl (100 mM final concentration) had been added. Reconstitution experiments using the supernatant were accomplished by dialysis of the supernatant against distilled water, lyophilization and redissolving the freeze-dried sample in 0.5 M sucrose, 5 mM $MgCl_2$, 5 mM sodium phosphate. The supernatant was added at a concentration equivalent to five times the bacteriochlorophyll present in the assay mixture.

Light-dependent alanine uptake was measured as described previously [7]. Spheroplasts or cells were present at a concentration equivalent to 150 μM bacteriochlorophyll and with 250 μM DCCD present to prevent any alanine uptake in the dark [7]. All light-dependent uptake was measured in 50 mM potassium phosphate buffer at pH 6.6, the pH optimum for uptake. Uptake experiments with spheroplasts were all performed in the presence of 0.5 M sucrose. Saturating light intensity ($7.5 \cdot 10^4$ ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}) was used. Alanine

uptake after 3 min illumination was used as a measure of the uptake rate. As the uptake rate by intact cells was always linear for more than 5 min, such rates were suitable for K_m determinations. Alanine uptake in the dark, driven by a K^+ plus valinomycin-induced membrane potential [9] and photophosphorylation [10] were measured as described previously.

Light intensities were measured with a Yellow Springs Instruments Model 65A radiometer. Absorbance spectra were obtained using an Aminco DW-2a spectrophotometer. Bacteriochlorophyll was measured as described by Clayton [11]. Protein was determined using the method of Bradford [12]. Gram staining was performed as described by Benson [13].

Azurin and cytochrome *c*-551 from *P. aeruginosa* were prepared in the laboratory of Dr. Harry B. Gray at the California Institute of Technology. Spinach plastocyanin was released from spinach leaves and purified through the $(NH_4)_2SO_4$ precipitation step as described by Borchert and Wessels [14]. The purification was completed according to the method of Yocum et al. [15]. High-potential iron protein (Hipip) was isolated from *C. vinosum* and purified as described by Bartsch [16,17]. Cytochrome *c*-551, cytochrome *c'*, cytochrome '*f*' (cytochrome *c*-553(550)) and the flavocytochrome *c*-552 were purified using a modification of the procedure of Bartsch [17]. The ferredoxin-free extract was subjected to $(NH_4)_2SO_4$ precipitation with cytochrome *c*-551 and cytochrome *f* being found in the 20—55% $(NH_4)_2SO_4$ precipitate and cytochrome *c'* and cytochrome *c*-552 being found in the 55—100% $(NH_4)_2SO_4$ precipitate. After dialysis, the $(NH_4)_2SO_4$ fractions were chromatographed on DEAE-cellulose as originally described by Bartsch [17]. The four cytochromes were then further purified by chromatography on Sephadex G-75. The absorbance spectrum of all proteins used in this study were essentially identical to those in the literature. Protein concentrations were determined from published extinction coefficients. It was assumed that ϵ for the Soret band of oxidized cytochrome *c*-551 was 180 $mM^{-1} \cdot cm^{-1}$.

Albumin (bovine serum), equine heart cytochrome *c*, lysozyme, valinomycin, alanine, ADP, and carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) were obtained from Sigma Chemical Company and used without further purification. [^{14}C]Alanine (6.216 TBq/mol) was obtained from New England Nuclear.

Results

Our previous experiments on alanine uptake by whole *C. vinosum* cells demonstrated that the uptake was energy dependent in that either light or the ability to hydrolyze ATP was required for uptake [7]. In order to maximize the possibility that the measured alanine uptake represented alanine transport, free from subsequent metabolism of alanine by *C. vinosum* cells, *N,N'*-dicyclohexylcarbodiimide (DCCD) was added [7] and illumination times were kept short. Fig. 1 shows that under these conditions, essentially all of the ^{14}C-labeled alanine taken up could be exchanged for excess unlabeled alanine and was released when an uncoupler such as CCCP was added. The alanine transport system followed Michaelis-Menten kinetics with K_m = 16 μM.

In order to examine the possible role of soluble electron carriers in the elec-

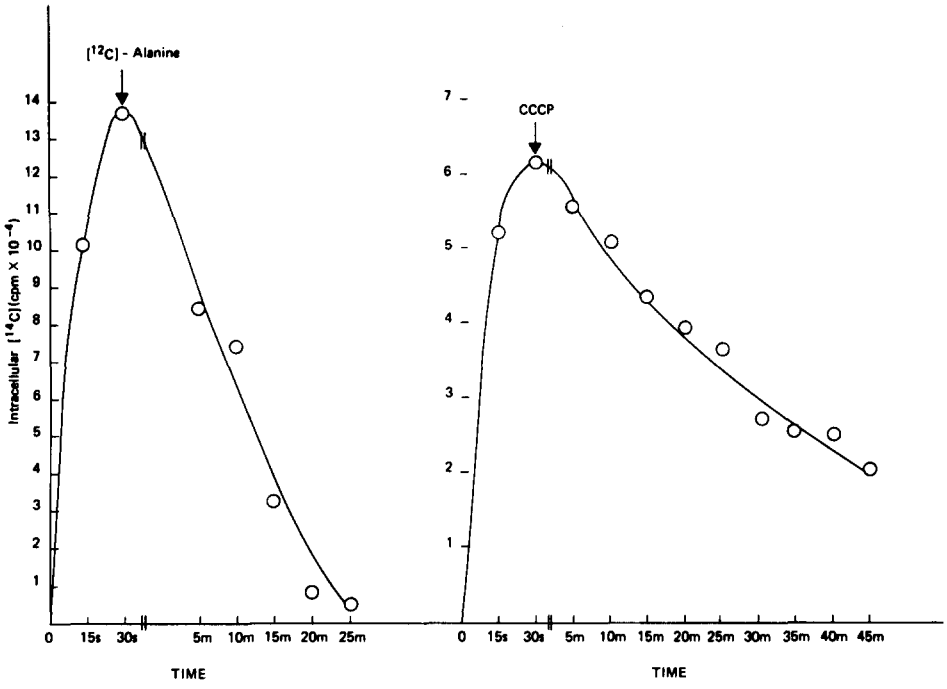

Fig. 1. Effect of alanine and CCCP on the steady-state intracellular level of [^{14}C]alanine in illuminated *C. vinosum* cells. The reaction mixture contained, in addition to the components described in Methods, 20 μM [^{14}C]alanine (specific activity 6.216 TBq/mol). At the indicated times [^{12}C]alanine final concentration 2 mM or CCCP final concentration 30 μM was added.

tron transport that provides the energy for light-dependent alanine uptake, it was necessary to prepare spheroplasts (free of cells walls) from *C. vinosum*. Treatment of *C. vinosum* cells with lysozyme plus EDTA, as described in Methods, does produce such spheroplasts. After treatment with lysozyme the characteristic Gram-negative staining pattern [13] seen with intact *C. vinosum* cells was lost. Suspension of the lysozyme-treated cells in hypotonic medium resulted in lysis (observed with a microscope), while untreated *C. vinosum* cells showed little or no lysis. Furthermore, the supernatant (after centrifugation at 145 000 × *g* for 30 min) obtained from suspending lysozyme-treated cells in distilled water contained large amounts of soluble protein, while a similar supernatant obtained from control *C. vinosum* cells showed no detectable protein. These *C.vinosum* spheroplasts retained a considerable portion of the DCCD-insensitive, light-dependent alanine uptake capability of *C. vinosum* cells. This uptake showed pH and light intensity dependencies similar to those of *C. vinosum* cells and was inhibited by 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO) and uncouplers over the same concentration ranges found with intact cells. Results similar to those shown in Fig. 1 were also obtained with spheroplasts, suggesting that (in the presence of DCCD) alanine uptake during the first 3 min of illumination is accompanied by little subsequent alanine metabolism. Alanine uptake by the spheroplast preparations was generally not sustained for illumination periods longer than 5 min. This, and

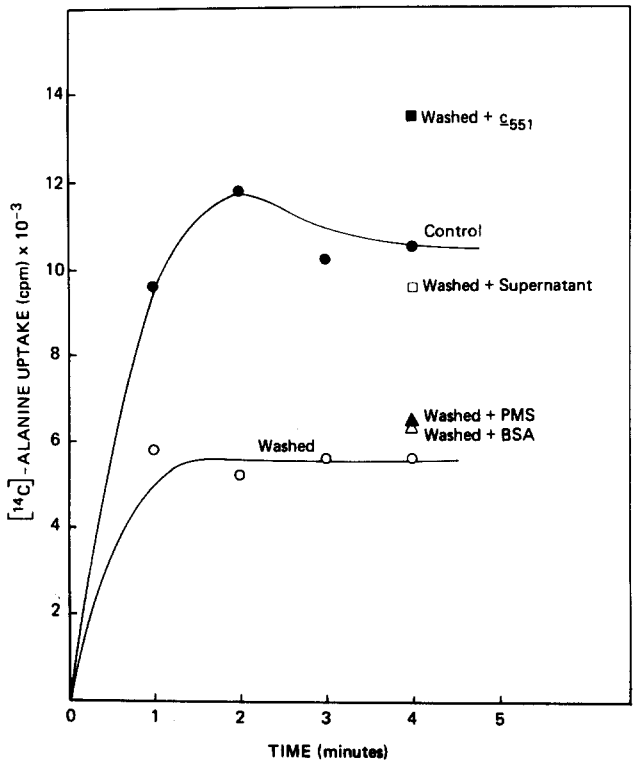

Fig. 2. The effect of washing and cytochrome *c*-551 on alanine uptake by illuminated *C. vinosum* spheroplasts. Reaction conditions as in Fig. 1, except that cells were replaced by spheroplasts. Bovine serum albumin (BSA), *N*-methylphenazonium methosulfate (PMS) and *C. vinosum* cytochrome *c*-551 were present at a concentration of 5 μM. Addition of concentrated supernatant was as described in Methods.

the fact that alanine uptake was not perfectly linear with time, precluded accurate K_m determinations for alanine uptake by the spheroplasts. However, it was estimated that $K_m < 60$ μM for these preparations.

Fig. 2 shows that washing the *C. vinosum* spheroplasts in isotonic sucrose containing 100 mM KCl resulted in a large decrease in the rate of light-dependent alanine uptake. Two washes were only marginally more effective than one in decreasing the rate of alanine uptake. Washing with isotonic sucrose containing 100 mM KCl was more effective than sucrose alone in decreasing alanine uptake but further increases in KCl concentration had no additional effect. Washing had no effect on alanine uptake in the dark caused by a K^+ plus valinomycin-induced membrane potential ($\Delta\psi$), as alanine uptake by washed spheroplasts was always at least 90% that of unwashed spheroplasts. This $\Delta\psi$ (exterior positive)-driven alanine uptake [9] provides an assay for the transport system alone, as energy for uptake is supplied directly by $\Delta\psi$. Thus, washing apparently had no effect on the alanine transport system itself.

Fig. 2 also shows that concentrated supernatant from the washing could restore light-dependent alanine uptake. Addition of bovine serum albumin to washed spheroplasts had no effect on light-dependence alanine uptake, suggesting that restoration of alanine uptake by the supernatant is not due to some

TABLE I

THE EFFECT OF *C. VINOSUM* ELECTRON CARRIERS ON ALANINE UPTAKE BY ILLUMINATED, WASHED SPHEROPLASTS

Reaction conditions were as described in Fig. 2. Hipip was present at a concentration of 5 μM and all other electron carriers were present at a concentration of 10 μM. The values for cytochromes *c*-552, *c'* and *f* represent averages of two determinations. The relative alanine uptake by unwashed spheroplasts averaged 4.0 ± 0.4 for this set of three experiments. The uptake by spheroplasts supplemented with cytochrome *c*-551 corresponds to 225 pmol of alanine.

Additions to washed spheroplasts	Relative light-dependent alanine uptake
None	1.00
Cytochrome *c*-552	1.59
Cytochrome *f*	1.34
Cytochrome *c'*	1.62
HiPIP	0.80
Cytochrome *c*-551	10.1

nonspecific protein effect. Since the alanine transport system was not affected by the washing procedure, it seemed likely that the component removed by washing participates in the light-driven cyclic electron flow known to provide the energy for light-dependent alanine uptake [7]. The ability of several purified soluble *C. vinosum* electron carriers in restoring light-dependent alanine uptake by washed spheroplasts was thus tested. Because cytochrome *c*-551 had been implicated as an electron carrier in the *C. vinosum* cyclic system by the work of van Grondelle et al. [6], it was tested first. Fig. 2 shows that cytochrome *c*-551 addition to washed spheroplasts increases the amount of alanine taken up after 4 min of illumination 2.4-fold. The non-physiological electron carrier, *N*-methylphenazonium methosulfate [18], was ineffective. Similar results were obtained using 2-min illumination times.

In order to test the specificity of the restoration of light-dependent alanine transport by cytochrome *c*-551, several other electron carriers isolated from *C. vinosum* were tested. The results of three such experiments are summarized in Table I. As can be seen, a large stimulation was obtained with cytochrome *c*-551 and little or no stimulation was obtained with the other electron carriers. Although there was considerable variability in the loss of alanine uptake on washing with different spheroplast preparations used over the course of this study, the only soluble electron carrier from *C. vinosum* that gave a substantial stimulation of light-driven alanine uptake in any experiment was cytochrome *c*-551.

As a further test of the specificity of cytochrome *c*-551 in the cyclic electron transport system of *C. vinosum*, several electron carriers isolated from other organisms were tested. Fig. 3 shows the results of two such experiments. Of all the electron carriers tested, only mitochondrial (equine) cytochrome *c* and cytochrome *c*-551 from *P. aeruginosa* could replace the native *C. vinosum* cytochrome *c*-551. In general, the *P. aeruginosa* cytochrome was as effective as the *C. vinosum* cytochrome while the equine cytochrome *c* gave even higher rates of alanine uptake than did the native cytochrome *c*-551. It has been demonstrated by X-ray crystallography that soluble cytochrome c_2 from the

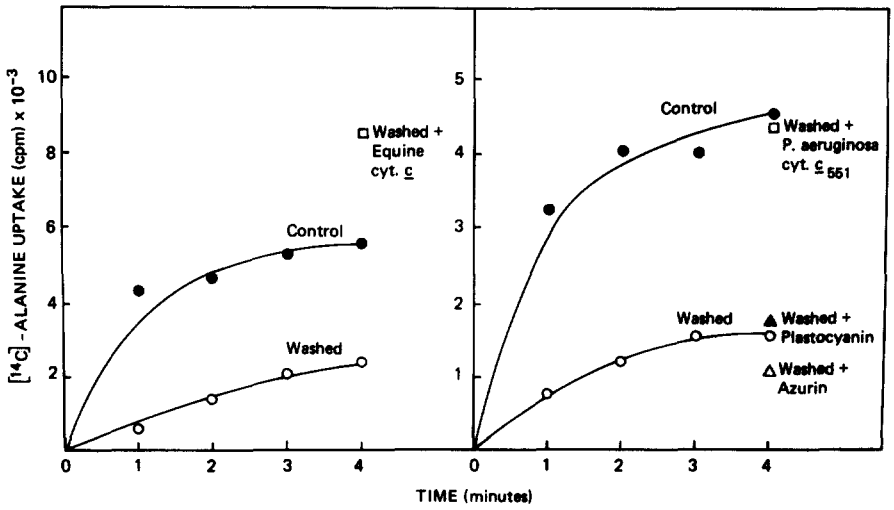

Fig. 3. The effect of added electron carriers on alanine uptake by washed *C. vinosum* spheroplasts. Reaction conditions were as in Fig. 3 with all added electron carriers present at a concentration of 5 μM. ●, control (unwashed) spheroplasts; ○, washed spheroplasts; □, washed spheroplasts to which equine cytochrome *c* (cyt. *c*) (left) or *P. aeruginosa* cytochrome *c*-551 (right) was added; △, washed spheroplasts to which *P. aeruginosa* azurin was added; ▲, washed spheroplasts to which spinach plastocyanin was added.

photosynthetic purple non-sulfur bacterium *Rhodospirillum rubrum* is related to mitochondrial cytochrome *c* (equine and tuna heart) and to *P. aeruginosa* cytochrome *c*-551 [19]. The results in Fig. 3 suggest a similar relationship may exist for *C. vinosum* cytochrome *c*-551.

The ability of *C. vinosum* cytochrome *c*-551 to restore light-dependent alanine uptake in washed *C. vinosum* spheroplasts suggested that similar results might be obtained with light-dependent ATP formation. However, no detectable photophosphorylation could be observed with control or washed spheroplasts. Neither the addition of *C. vinosum* cytochrome *c*-551, nor of equine cytochrome *c*, nor of the non-physiological electron carrier *N*-methylphenazonium methosulfate resulted in any detectable ATP production from ADP and phosphate by illuminated *C. vinosum* spheroplasts.

Discussion

Previous work in our laboratory demonstrated that light-dependent alanine uptake by *C. vinosum* cells is inhibited by HOQNO [7], an inhibitor known to block cyclic electron flow in this bacterium [6,18]. Furthermore, the concentration dependence of HOQNO inhibition of alanine uptake [7] matched that of its inhibition of cyclic electron flow [6]. Thus, light-dependent alanine uptake in *C. vinosum* can be used as a measure of cyclic electron flow by this bacterium. The demonstration of a light-dependent, HOQNO-sensitive alanine uptake by *C. vinosum* spheroplasts indicated that the spheroplasts retain some ability to perform light-driven cyclic electron flow. Since washing severely inhibits light-dependent alanine uptake by *C. vinosum* spheroplasts without any inhibition of the transport system per se (measured by $\Delta\psi$-induced alanine

uptake), it can be concluded that the cyclic electron transport chain contains a soluble component that is exposed upon loss of the cell wall. As the only known soluble electron carrier isolated from *C. vinosum* that restored light-dependent alanine uptake was cytochrome *c*-551, it appears highly likely that this cytochrome participates in cyclic electron flow. Our data thus fully support the role for cytochrome *c*-551 in *C. vinosum* proposed by van Grondelle et al. [6]. The fact that cytochrome *c*-551 can be washed from the cell membrane of *C. vinosum* spheroplasts suggests that the in vivo location of this cytochrome is in the periplasmic space between the cell membrane and the cell wall. Similar locations for the soluble photosynthetic cytochromes c_2 from the purple non-sulfur bacteria *Rhodopseudomonas sphaeroides* [20] and *Rhodopseudomonas capsulata* [20—22] have been demonstrated previously. The observation that addition of cytochrome to washed spheroplasts often gave alanine uptake rates greater than those obtained with control (unwashed spheroplasts) suggests that considerable cytochrome *c*-551 is lost during spheroplast preparation.

A recent review by Salemme [19] has pointed out the fundamental similarities between the three-dimensional structures (and presumably, oxidation-reduction mechanisms) of the cytochromes *c* from mitochondria (tuna and equine), from the photosynthetic bacterium *R. rubrum* and from *P. aeruginosa*. These similarities in overall tertiary structure and, in particular, in the heme environment have been made even more apparent by the recently determined high-resolution structure of the *P. aeruginosa* cytochrome [23]. The fact both mitochondrial (equine) cytochrome *c* and *P. aeruginosa* cytochrome *c*-551 can substitute for the native *C. vinosum* cytochrome *c*-551 in cyclic electron flow suggests that *C. vinosum* cytochrome *c*-551 belongs to the same evolutionary family as these other *c*-type cytochromes. The ineffectiveness of azurin, isolated from the same organism as *P. aeruginosa* cytochrome *c*-551 provides a partial control for the specificity of the system as does the ineffectiveness of spinach plastocyanin. The fact that plastocyanin does not substitute for *C. vinosum* cytochrome *c*-551 is of interest because plastocyanin can replace a soluble *c* cytochrome in some algal systems [24].

Our failure to observe photophosphorylation with *C. vinosum* spheroplasts even though light-driven cyclic electron flow is occurring was not surprising. *C. vinosum* cells and spheroplasts are known to have the opposite sidedness of *C. vinosum* chromatophores [10]. Antibody experiments have demonstrated that *C. vinosum* ATPase (the enzyme that catalyzes ATP formation) is located on the outside of chromatophores [25]. Thus, ADP and inorganic phosphate added to the reaction mixture would not be accessible to ATPase located on the inside of the spheroplast membrane unless transport systems for ADP and phosphate were present in the *C. vinosum* membrane. Under the preparative conditions used in these experiments, it is unlikely that internal phosphate would be limiting. Thus the absence of detectable photophosphorylation by *C. vinosum* spheroplasts suggests that this bacterium does not possess a highly active adenine nucleotide transport system, such as that found in the purple non-sulfur bacterium *Rps. capsulata* [26]. Cytochrome *c*-551 is likely to be required for light-dependent ATP formation by *C. vinosum* (as is the soluble cytochrome *c*-420 in *R. rubrum* [27]) and this likelihood is currently under further investigation.

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