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PROTON UPTAKE AND QUENCHING OF BACTERIOCHLOROPHYLL FLUORESCENCE IN *RHODOPSEUDOMONAS SPHEROIDES*

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SUMMARY

The addition of the cyclic cofactor 2,3,5,6-tetramethyl-*p*-phenylenediamine (diaminodurene) to a suspension of chromatophores of *Rhodopseudomonas spheroides* causes a light-dependent quenching of bacteriochlorophyll fluorescence. This effect is similar to one observed in chloroplasts and related to proton uptake. It is distinct from the quenching operative through the redox state of the primary electron donor and acceptor, as shown by its sensitivity to uncouplers and ionophorous antibiotics. The quenching is dependent on light intensity and diaminodurene concentration, and has a pH optimum at 7.1 where up to 70 % of the fluorescence could be quenched in the presence of 0.33 mM diaminodurene.

Measurements of this light-induced fluorescence quenching and of light-induced proton uptake were performed on similar solutions containing diaminodurene and various uncouplers and ionophorous antibiotics. All of these substances affected the fluorescence quenching and proton uptake in a similar fashion. The nigericin-like antibiotic, X-464, inhibited the proton uptake by 85 % and reversed or prevented at least 95 % of the fluorescence quenching. Qualitatively similar results were obtained with NH_4Cl, $(NH_4)_2SO_4$ and carbonylcyanide 3-chlorophenylhydrazone (CCCP). However, those compounds such as valinomycin and gramicidin which cause an increase in the rate of proton uptake, precipitated a rapid quenching of fluorescence, which differed kinetically from the usual quenching. It is concluded from these studies that the fluorescence quenching is related to the uptake of protons, and may be useful as a probe to monitor proton accumulation in chromatophores.

INTRODUCTION

Measurement of chlorophyll and bacteriochlorophyll fluorescence, both *in vivo* and *in vitro*, has been an important tool for elucidating the nature of the primary

Abbreviations: diaminodurene, 2,3,5,6-tetramethyl-*p*-phenylenediamine; CCCP, carbonylcyanide 3-chlorophenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PMS, phenazine methosulfate; HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; MES, (*N*-morpholino)-ethanesulfonic acid.

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events in photosynthesis[1–3]. The use of fluorescence has been extended by employing fluorescent molecules that are responsive to environmental changes in specific ways[4]. In particular, the quenching of atebrin fluorescence can be related to the light-induced proton uptake and has been used as a measure of the pH gradient in chloroplasts[5,6]. A drawback of these techniques is the possibility of perturbing the membrane environment that is under study. However, recent work has shown that the chlorophyll *a* fluorescence itself can be used to probe the high-energy state in chloroplasts. Addition of cofactors of cyclic electron flow, such as 2,3,5,6-tetramethyl-*p*-phenylenediamine (diaminodurene)[7] or phenazine methosulphate (PMS), catalyzes a light-induced, energy-dependent quenching of chlorophyll *a* fluorescence[8–10]. This quenching is independent of changes in the redox state of the usual quencher of Photosystem II fluorescence, and is reversed on the addition of uncouplers. It has been shown that this fluorescence quenching can be related to proton uptake[9,10], and may therefore be a reflection of the high-energy state. In the present work, we have extended these studies to the photosynthetic bacterium, *Rhodopseudomonas spheroides*, and have found that the addition of diaminodurene causes a large light-induced quenching of bacteriochlorophyll fluorescence. We will show that this fluorescence quenching can be related to the uptake of protons in the light, and we will discuss these findings with respect to the mechanism of energy coupling in photosynthetic bacteria.

MATERIALS AND METHODS

Rhodopseudomonas spheroides 2.4.1 (van Niel) was grown photosynthetically in modified Hutner medium as described previously[11]. The cells were harvested by centrifugation, and resuspended in 10 mM Tricine buffer containing 0.25 M sucrose (pH 7.4). Chromatophores were prepared by the method of Worden and Sistrom[12] and were finally resuspended in 0.25 M sucrose after the second high speed centrifugation. Though light chromatophores[12] were generally used, cruder extracts gave identical results. Chromatophores were stored under argon at 4 °C at 50 $A_{850\,nm}$/ml for no more than 48 h. The bacteriochlorophyll concentration was determined according to Clayton[13].

Reaction centers from *R. spheroides* were prepared by Dr S. C. Straley by the method of Clayton[14]. Reaction centers were adjusted to a final concentration of 0.2 $A_{870\,nm}$/ml in 0.01 M Tris (pH 7.5) and 0.10–0.12 % lauryl dimethyl amine oxide. The concentration of diaminodurene used in these experiments varied between 2–60 μM. The measurement of reaction center fluorescence and absorption changes were performed as described by Clayton *et al.*[15].

The non-photosynthetic mutant of *R. spheroides*, strain PM-8, was isolated by Clayton and Wang[14]. This mutant contains a normal complement of light-harvesting bacteriochlorophyll and carotenoids, but lacks reaction centers[17,18] and P870[19]. Since PM-8 must be grown aerobically in the dark, *R. spheroides* strain Ga (the photosynthetically competent parent of PM-8) grown in this fashion was used as a control. It gave results comparable to the wild type.

The fluorimeter used to measure the light-induced fluorescence changes has been recently described in detail[10]. The amplified signals were recorded on a Bausch and Lomb VOM 5 recorder and a Hewlett–Packard 141 A oscilloscope. The reaction mixture for the fluorescence measurements usually contained (in 3 ml): 100 mM KCl,

20 mM (*N*-morpholino)ethanesulfonic acid (MES) (pH 5.5-7.0) or 20 mM Tricine (pH 7.1–8.5), and chromatophores at a concentration of 5 μg bacteriochlorophyll/ml. Unless indicated, diaminodurene was present at a final concentration of 0.33 mM. Exciting light was filtered through a Baird-Atomic 800 nm interference filter and the fluorescence was measured through a 902 nm filter in conjunction with Kodak Wratten 87B and 87C filters. The experiments were performed by first measuring the fluorescence of chromatophores alone. The light was then turned off, diaminodurene and other compounds were added, and the light was once more turned on. The light-induced quenching was then followed for 2 min. When diaminodurene was injected during illumination, the same level of quenching was reached.

Light-induced proton uptake was measured as described elsewhere[20]. Chromatophores for these experiments were suspended in 100 mM choline chloride + 5 mM Tricine, pH 7.4, at a concentration of 250 μg bacteriochlorophyll/ml. The reaction mixture contained (in 4 ml): 100 mM KCl or choline chloride, 100 μM diaminodurene and chromatophores equivalent to 100 μg bacteriochlorophyll. At this dilution of the chromatophore suspension, the reaction mixture contained 0.5 mM Tricine. The starting pH was adjusted to 6.4–6.5. The actinic light was passed through Corning 2-59 and 7-69 filters and the intensity incident on the sample was equal to 12 mW/cm^2.

Diaminodurene, prepared according to Michaelis *et al.*[21], was generously supplied by Dr R. E. McCarty. The nigericin-like antibiotic, X-464, and gramicidin were donated by Dr A. T. Jagendorf. Valinomycin and carbonylcyanide 3-chlorophenylhydrazone (CCCP) were purchased from Calbiochem, and PMS and 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) from Sigma. Diaminodurene is now available from Eastman Chemical.

RESULTS

Light-induced quenching of bacteriochlorophyll fluorescence in the presence of diaminodurene

The addition of diaminodurene to a suspension of chromatophores caused a slow quenching of fluorescence, a typical time course of which is seen in Fig. 1. When the light was turned on in the presence of diaminodurene, there was a rapid rise to the control level, followed by a fast quenching of a few percent (Figs 1C and 1D). There was then a plateau of about 15–30 s where little or no quenching occurred (Fig. 1A). Subsequent to this, there was a slow quenching of fluorescence which reached a steady state level in 2–4 min, depending on the preparation. To standardize the measurements, the percent quenching was determined after 2 min of illumination. At high light intensity (5–7 mW/cm^2), high diaminodurene concentration (0.33 mM) and at pH7.1, the maximal quenching observed was 60–70 %.

The extent of the fluorescence quenching was a hyperbolic function of the diaminodurene concentration, yielding a K_m of about 100 μM at high light intensities (Fig. 2). The extrapolated maximum quenching was between 70–75 % at high diaminodurene concentrations under these conditions. The quenching was light dependent and very slowly dark reversible. If, after 2 min illumination in the presence of diaminodurene, the suspension was allowed to sit in the dark for 5–10 min, a partial reversal of the quenching could be seen (50–75 % reversal). Quenching during the next illumination then proceeded more rapidly to the steady-state level. This effect is similar,

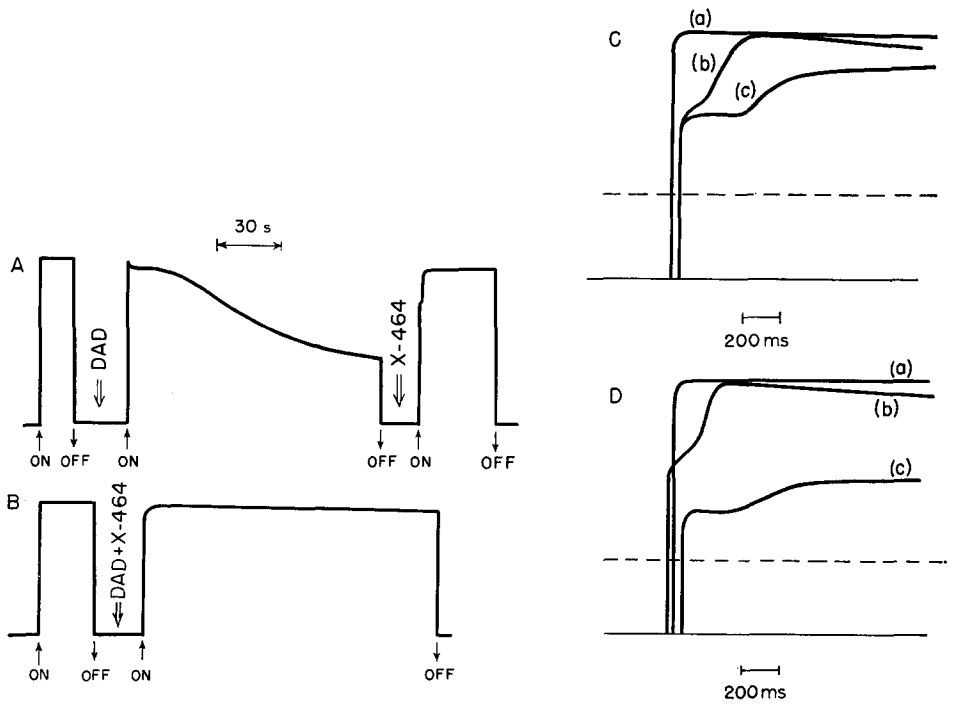
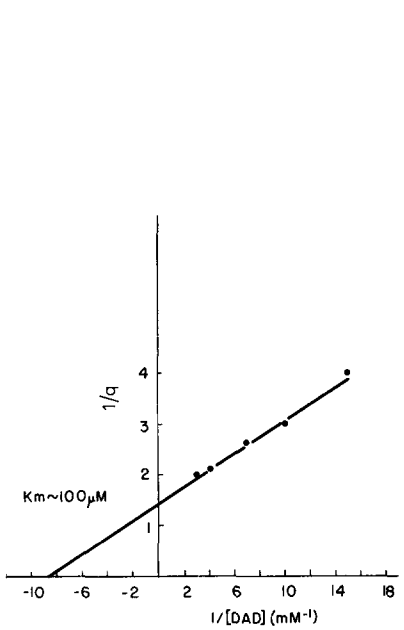


Fig. 1. Quenching of bacteriochlorophyll fluorescence in *R. spheroides* chromatophores. The clear-sided cuvette contained, in 3 ml, chromatophores at a concentration of 5 μg bacteriochlorophyll/ml in 100 mM KCl + 20 mM MES, pH 7.1. All additions were made in the dark. Light intensity (800 nm) = 7.1 mW/cm^2. (A) Light-induced quenching in the presence of 0.33 mM diaminodurene (DAD). Nigericin (0.2 μM X-464) added after completion of quenching. (B) Nigericin added at the same time as diaminodurene. (C) Oscilloscope tracing of experiments similar to those above. (a) Chromatophores; (b) +0.33 mM diaminodurene; (c) the quenching in (b) was allowed to continue until the level of the dotted line was reached. Nigericin (0.2 μM) was then added and the quenching was reversed. (D) Conditions were the same as above. (a) Chromatophores; (b) +0.33 mM diaminodurene; quenching reached the level of the dotted line after 2 min of illumination; (c) 0.33 mM diaminodurene +0.33 μM valinomycin added at the same time in the dark before illumination.

though not nearly as extensive, to that seen in chloroplasts[9,10]. The percent quenching was not affected materially by the addition of salts such as KCl, NaCl, or choline chloride, nor by the molarity of the cation present (1–200 mM).

The light-induced quenching of fluorescence in the presence of diaminodurene was dependent, though, on the external pH, as seen in Fig. 3A. The quenching was optimal at pH 7.1, though it was still above 40 % between pH 5.5–8.0. The kinetics of the quenching changed slightly at pH 8.0–8.5. In these cases, there was a rapid quenching of fluorescence in less than 200 ms which was about 10 % of the total. This was then followed by the usual slow quenching.

The dark reversal of the quenching also depended markedly on the pH. This reversal was slowest at pH 6.5–7.5, being in the range of 5 min for 50 % reversal. At alkaline pH, this increased rapidly, until at pH 8.5, nearly 100 % reversal could occur within 1 min. This should be compared with the dark reversal in chloroplasts, for which the half time decreased from 9 s at pH 7.5 to 1.5 s at pH 9.0[9].



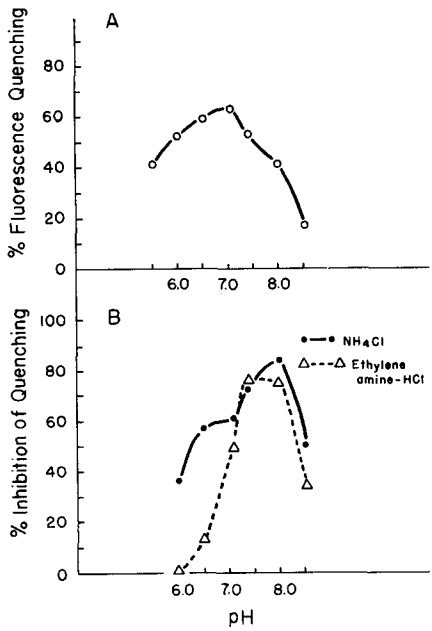


Fig. 2. Reciprocal plot of fluorescence quenching *vs* diaminodurene (DAD) concentration. Experimental conditions were identical to those of Fig. 1.

Fig. 3. (A) pH dependence of the light-induced quenching in the presence of diaminodurene. Chromatophores (5 μg bacteriochlorophyll/ml) were suspended in 100 mM KCl and 20 mM buffer, either MES (pH 5.5–7.1) or Tricine (pH 7.1–8.5). The percent quenching at pH 7.1 was the same in either buffer. Light intensity (800 nm) = 7.1 mW/cm^2. The diaminodurene concentration was 0.33 mM. (B) pH dependence of NH_4Cl and ethyleneamine inhibition of quenching. Conditions were the same as above, except that 6.0 mM NH_4Cl or 6.0 mM ethyleneamine·HCl were added at the same time as diaminodurene and the light-induced quenching measured.

The light-induced fluorescence quenching was almost entirely inhibited in the presence of 0.33 mM *o*-phenanthroline, a compound which presumably blocks electron flow between the primary and secondary electron acceptors[23]. This implies that electron transport is necessary to elicit the diaminodurene-catalyzed quenching. The addition of HQNO[24] inhibited the quenching at concentrations above 10 μM.

Effect of diaminodurene on the fluorescence of reaction centers and a reaction center-less mutant

Fluorescence studies in *R. spheroides* can be extended by the preparation of particles that, *in vitro*, can carry out the primary photochemistry. Therefore, it is possible to study the fluorescence of reaction center bacteriochlorophyll[15] separately from that derived from the bulk bacteriochlorophyll[16]. Using reaction centers, we can gain insight into the effect of diaminodurene on the redox state of the primary donor and primary acceptor, and the consequent implications for bacteriochlorophyll fluorescence. Alternatively, using a photosynthetic mutant of *R. spheroides* that lacks reaction center bacteriochlorophyll P870[16–18], it is possible to determine if the fluorescence quenching is a property of the bulk pigments.

The addition of 2–60 μM diaminodurene to a suspension of reaction centers

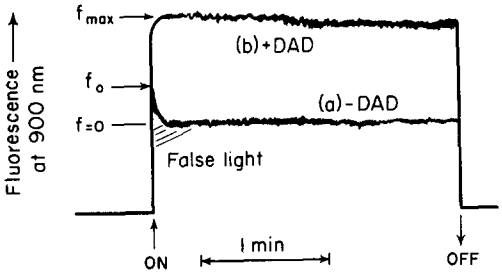


Fig. 4. Time course of fluorescence at 900 nm from *R. spheroides* reaction centers. The reaction centers at 1.8 μM (ε = 113 $mM^{-1} \cdot cm^{-1}$ at 867 nm) were suspended in 10 mM Tris, pH 7.5, with 0.12 % lauryl dimethyl amine oxide. The exciting light, through an 800 nm interference filter, was 4.5 mW/cm^2. (a) No electron donor added. (b) +4 μM diaminodurene (DAD). See Clayton[15] for detailed explanation of this type of experiment.

caused a large increase in fluorescence (Fig. 4). The kinetics are identical to those shown in Clayton *et al.*[15], Fig. 1. Clayton has inferred[15,25], that a fluorescence change of this type is indicative of a substance acting as an electron donor. The presence of a suitable electron donor prevents the accumulation of oxidized P870 while allowing photochemical reduction of the primary acceptor: P870,A $\xrightarrow{\text{light}}$ P870,A^-. The state P870,A^- is more strongly fluorescent than P870,A. The increase in fluorescence was a factor of 3–4, about the same as that seen in the presence of reduced cytochrome *c* or dithionite[15]. After about 10 s of illumination, some fluorescence lowering was seen, presumably due to some oxidized diaminodurene acting as an electron acceptor and pulling electrons from A^-. However, this 2–5 % quenching still left the fluorescence 3-fold higher than before the addition of diaminodurene. That diaminodurene prevented the sustained bleaching of P870 was shown by measurements of the light-induced absorbance changes at 863 nm. Without diaminodurene, light converted P870 to P^+870 and P870 remained bleached in the light. When diaminodurene was present, the P^+870 formed in the light was reduced to P870 with a $t_{1/2}$ = 2 s at this concentration of diaminodurene. Uncouplers or ionophorous antibiotics had no effect on these fluorescence phenomena.

The addition of diaminodurene (at concentrations 2–500 μM) had no effect on the fluorescence from chromatophores of the non-photosynthetic mutant PM-8. This is consistent with the lack of any effect of other donors on PM-8[16], and shows that the diaminodurene-catalyzed fluorescence quenching is not due to some extraneous effect on the bulk bacteriochlorphyll. Chromatophores prepared from *R. spheroides* Ga, grown aerobically in the dark, showed fluorescence quenching effects comparable in magnitude to those seen in the photosynthetically grown wild type.

Effect of uncouplers and ionophorous antibiotics

The slow fluorescence quenching in the presence of diaminodurene was highly sensitive to uncouplers and ionophorous antibiotics, the effects of which fell into two distinct classes. Those substances, such as ammonium salts, amines, and the nigericin-like antibiotic, X-464, which specifically effect proton movements[26–28] reversed the slow quenching in a characteristic way. The antibiotics valinomycin and gramicidin, which affect the membrane potential as well as proton movements[26], caused a partial, very rapid quenching of fluorescence that could be followed by the typical slow quenching.

TABLE I

EFFECT OF INHIBITORS ON FLUORESCENCE QUENCHING AND PROTON UPTAKE

Fluorescence quenching and proton uptake were measured under similar conditions. For the quenching experiments, the cuvette contained chromatophores (5 μg bacteriochlorophyll/ml), 100 mM KCl, 20 mM MES, pH 6.5, and 0.33 mM diaminodurene. $I = 7.1$ mW/cm^2. The proton uptake reaction mix contained chromatophores (25 μg bacteriochlorophyll/ml), 100 mM KCl, 0.5 mM Tricine, and 100 μM diaminodurene. The final pH was 6.4–6.5. Where indicated, 10 mM KCl or 100 mM choline chloride was substituted for 100 mM KCl. The final concentration of the inhibitors were: nigericin, 0.2 μM; NH_4Cl or $(NH_4)_2SO_4$, 6.0 mM; CCCP, 10 μM; valinomycin, 0.33 μM.

Additions	*Quenching* (%)	*Inhibition* (%)	*pH shift*			
			Initial rate		*Extent*	
			μequiv H^+ accumulated/mg bacteriochlorophyll/min	*Inhibition* (%)	*μequiv H^+ accumulated/mg bacteriochlorophyll*	*Inhibition* (%)
None	62	—	3.20	—	1.06	—
Nigericin	7	89	0.78	76	0.16	85
Nigericin (10 mM KCl)	15	75	1.03	68	0.30	72
Nigericin (100 mM choline chloride)	27	57	1.22	62	0.53	50
NH_4Cl	26	58	1.60	50	0.56	47
NH_4Cl (100 mM choline chloride)	27	56	1.92	40	0.56	47
$(NH_4)_2SO_4$	30	52	1.95	39	0.56	47
$(NH_4)_2SO_4$ (100 mM choline chloride)	22	64	1.95	39	0.45	58
CCCP	6	90	1.28	60	0.32	70
Valinomycin	61	—	10.10	—	1.28	—

When the light-induced quenching of fluorescence was allowed to continue for 2 min and 0.2 μM nigericin was added in the dark, we obtained a near total reversal of quenching during the subsequent illumination (Fig. 1B). This effect, which occurred at all pH values between 5.5–8.5, was cation dependent, being more efficient in 100 mM KCl than in 100 mM choline chloride (Table I). Under conditions of maximal quenching, nigericin could reverse or prevent 85–100 % of the quenching (Table I).

Ammonium salts and amines had similar effects on the prevention of quenching, but had only a marginal effect on the reversal of quenching that was already fully developed. The effect of these compounds was pH dependent, with the maximal inhibition of the slow fluorescence quenching occurring at pH 7.5–8.0 (Fig. 3B). The results are in agreement with the findings of Crofts[29] in his study of amine uncoupling of energy transfer in chloroplasts. He has suggested that NH_3 is the active species in NH_4^+ uptake. Upon acidification of the chromatophores following H^+ uptake, the equilibration of NH_3 across the membrane would be displaced, and more NH_3 would enter the chromatophores to replace the lost by association with H^+.

The addition of valinomycin or gramicidin caused different changes in the bacteriochlorophyll fluorescence quenching. The addition of 0.33 μM valinomycin into a suspension where maximal quenching had already occurred caused some reversal of quenching (approx. 20 %). However, when valinomycin was added with diaminodurene in the dark, the fluorescence was observed to be less intense as soon as the light was turned on (Fig. 1D). The lowering of the initial fluorescence was pH dependent, being maximal (approx. 35 %) at pH 7.1 and varying between 15–25 % at other pH values. In all cases, this was followed by a slow quenching that reached the same level as that obtained in the absence of valinomycin. When valinomycin was injected in the light after the diaminodurene-catalyzed quenching had commenced, there was a slight increase in the rate of quenching but no change in extent. This lowering of the initial fluorescence also occurred with valinomycin in the presence of either nigericin or NH_4Cl under conditions that totally block phosphorylation[26–28,30]. Gramicidin acted very much like valinomycin.

The dark reversibility of the fluorescence quenching was markedly increased when valinomycin was present. This dark reversal of the quenched state had a $t_{1/2}$ = 30 s at pH 7.1 in the presence of valinomycin instead of the 5 min seen in its absence. When the light was turned on a second time, the quenced state was reached much more rapidly, with a $t_{1/2}$ = 3 s. This pattern was even more apparent at higher pH values and at pH 8.0, the dark reversal had a half time of 15 s, while the quenching on the second illumination was complete within 3 s.

Proton uptake studies

The effect of ammonium salts and ionophorous antibiotics strongly suggest that the light-induced fluorescence quenching in the presence of diaminodurene can be related to light-induced proton movements. Though numerous studies on proton uptake in chromatophores have been published[26,31–33], no systematic studies have been done on preparations from *R. spheroides*. Many of the difficulties in this system could be overcome using diaminodurene as a mediator, and we were able to measure H^+ uptake under conditions similar to those used to determine fluorescence quenching.

It was found that a good correlation could be made between the fluorescence quenching and the proton uptake.

When the light-induced proton uptake in *R. spheroides* chromatophores was measured in the absence of any cofactor, only a small amount of protons accumulated, generally less than 0.1 μequiv H^+ accumulated per mg bacteriochlorophyll. Addition of PMS or PMS + ascorbate + HQNO[24], at a variety of concentrations, increased the extent of proton uptake 2–3-fold. This was about the same increase that was seen upon the addition of succinate (see also ref. 34). However, a stimulation of better than 10-fold was seen in the presence of diaminodurene, yielding an extent of proton uptake of 1.0–1.4 μequiv H^+ accumulated per mg bacteriochlorophyll. This pattern differs substantially from that found using *R. rubrum* chromatophores, where high levels of proton uptake are found even in the absence of cofactor[31].

The kinetics of the light-induced proton uptake can be seen in Fig. 5. Protons

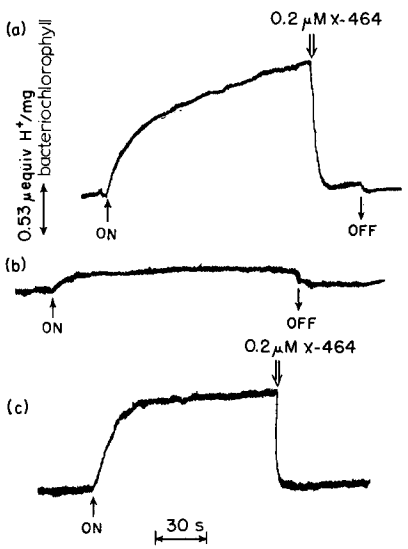


Fig. 5. Proton uptake in chromatophores of *R. spheroides*. The reaction mix contained (in 4 ml): 25 μg bacteriochlorophyll/ml, 100 mM KCl, and 0.1 mM diaminodurene at a starting pH of 6.4. (a) Control; (b) +0.2 μM nigericin; (c) +1 μM valinomycin.

could be taken up by the chromatophores in the light for up to 4–5 min, at which time a steady state was reached. As with the quenching, we computed the extent of H^+ uptake after 2 min of illumination, which was usually 70–90 % of the total extent. This uptake was dark reversible, but with a half time of about 5–10 min. The kinetics of dark decay were slowest at pH 6.0–6.5, and fastest at around pH 8.0, which closely resembles the kinetics of dark reversibility of fluorescence quenching.

The addition of nigericin to chromatophores in the light led to a near total efflux of the protons accumulated (Fig. 5a). When nigericin was present at the start of the light period, the uptake of protons was 75–90 % inhibited (Fig. 5b and Table I). In both cases the remaining protons that were accumulated were rapidly effluxed in the dark. The nigericin inhibition was partially dependent on K^+, a result similar to that seen in *R. rubrum*[26] (see also ref. 35 for X-464 specificity). Ammonium salts

and CCCP were also studied and gave the expected results (Table I). The addition of 0.33 mM *o*-phenanthroline inhibited at least 80 % of the H^+ uptake.

The addition of valinomycin or gramicidin, which were shown to cause an immediate lowering of the initial fluorescence, led to a faster initial uptake of protons. This is even true when valinomycin was added in the presence of nigericin or NH_4Cl. However, the lowering of the initial fluorescence was a much faster process, being complete in less than 20 ms. It is possible that the addition of valinomycin in the dark causes changes which are manifest as soon as the light is turned on.

Quenching with other donor systems

Bacteriochlorophyll fluorescence could be lowered by the addition of other donors such as dichlorophenolindophenol and PMS, (both *plus* and *minus* ascorbate), but the quenching was either minimal or not reversed by uncouplers. High concentrations of PMS caused an extremely rapid quenching of about 50 %, that was reversed completely by ascorbate or dithionite but only a few percent by nigericin. It was also apparent at low light intensities. Presumably, this quenching operates through the redox states of the primary donor and acceptor, or is due to a static quenching of excited bacteriochlorophyll[8]. The addition of 10–20 μM PMS and 1.6 μM HQNO[24] gave rise to a light-dependent quenching that was uncoupler reversible. However, this was never more than 5–10 % of the total fluorescence. A fluorescence lowering in the presence of $1 \cdot 10^{-3}$–$1 \cdot 10^{-5}$ M dichlorophenolindophenol and ascorbate, which has been previously reported by Cellarius[36], was only marginally sensitive to uncouplers.

DISCUSSION

The light-induced quenching of bacteriochlorophyll fluorescence in the presence of diaminodurene appears to differ from that operating through the redox state of the primary electron donor and acceptor. This is exemplified by the sensitivity of the quenching to ionophorous antibiotics and uncouplers, and by the relationship between the quenching and H^+ uptake.

Furthermore, the characteristics of the light-induced quenching are extremely similar to that found in chloroplasts[8–10], where the effects of "Q"-type quenching can be more firmly ruled out. In chloroplasts, the diaminodurene or PMS-catalyzed quenching can be performed in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which blocks the oxidation of the primary acceptor, and makes fluorescence changes due to variations in the redox levels unlikely under the conditions of the experiment. Though this separation of variables is not possible in chromatophores, the similarity of the quenching in the two systems can allow us to presume that the same considerations apply.

The rapid transient changes in the fluorescence seen in Figs 1C and 1D undoubtedly reflect changes in the proportion of open to closed traps. These fast transients are always seen upon the addition of diaminodurene, and are usually complete within 200 ms. The time constants of these changes are appropriate, for the light intensity used in the experiments and for a reasonably high quantum efficiency, with a pool of oxidized diaminodurene being filled in the experiments of Curves b

and c, but not in that of Curve a. Importantly, these redox changes are seen even in the presence of the ionophorous antibiotic nigericin (Fig. 1C, Curve c).

The slower changes in the fluorescence level, what we have termed the fluorescence quenching, could still be due to some kind of slow drift in the redox poise with the proportion of open traps slowly increasing. This possibility is exceedingly difficult to rule out. We have obtained indirect evidence against it by measuring light-induced absorbance changes at 780 nm in the presence and absence of electron transport cofactors. The addition of either diaminodurene, PMS, or tetramethyl-*p*-phenyldiamine caused a rapid, 2-fold increase in the ΔA at 780 nm. In all cases this level was stable over the time period used to measure fluorescence quenching. These changes were sensitive to electron transport inhibitors such as antimycin and HQNO, but not to uncouplers such as nigericin or NH_4Cl. This implies that no slow redox changes were occurring after the addition of diaminodurene, and, furthermore, that diaminodurene had the same effect on the redox poise as PMS and tetramethyl-*p*-phenylenediamine, substances which do not elicit a light-induced fluorescence quenching. Finally, since nigericin prevented or reversed the slow quenching without eliminating the rapid induction effects, it seems likely that the slow quenching is related to H^+ uptake (and not the state of the traps), while the fast transients are monitoring the redox poise of the traps.

Electron transport also appears to be required for the quenching to occur, in chromatophores as well as chloroplasts. *o*-Phenanthroline, which blocks electron flow between the primary and secondary electron acceptors[23], almost totally inhibits the quenching at concentrations which prevent over 80 % of the H^+ uptake. This is consistent with diaminodurene acting as a mediator of cyclic electron flow. Of interest, however, is the relative effectiveness in producing fluorescence quenching between diaminodurene and PMS, which also catalyzes electron transport[24]. Only a small amount of the quenching seen in the presence of PMS was sensitive to uncouplers. Yet, PMS stimulates electron transport in *R. spheroides* chromatophores to the same extent as diaminodurene as measured by the bleaching of bacteriochlorophyll at 780 nm, and it does stimulate H^+ uptake 2–3-fold. In *R. rubrum* chromatophores, where H^+ uptake is high even in the absence of cofactor, PMS and diaminodurene have a similar effect on the number of protons accumulated, though only diaminodurene gives rise to a light-dependent fluorescence quenching (unpublished observations). This could mean that diaminodurene has other properties that enable it to elicit such a large fluorescence quenching. It was found that the extent of proton uptake was greatly increased in the presence of diaminodurene. Since diaminodurene has a pK_a of approx. 6.0, it could be acting to increase the internal buffering capacity of the chromatophores and, thereby, cause a larger accumulation of protons. It would be of interest to see if an uncoupler-sensitive fluorescence quenching could be produced in the presence of PMS and a compound, such as pyridine[37], which can cause a large internal accumulation of protons in chloroplasts. However, attempts to find an energy-dependent quenching with pyridine and PMS have been unsuccessful.

We have shown that the light-induced quenching in the presence of diaminodurene is closely related to the uptake of protons. According to the chemiosmotic hypothesis of Mitchell[38,39], energy coupling is dependent upon a gradient in the electrochemical activity of H^+ across a membrane. This gradient has two components, a

chemical term due to the uptake of protons (the pH gradient) and an electrical term due to charge differences across the membrane (membrane potential). It is now firmly established that ATP production in chloroplasts depends almost entirely on the chemical component, since elimination of the proton gradient prevents ATP synthesis[5,40,41]. However, the opposite seems to be true in photosynthetic bacteria, since abolition of the proton gradient has little effect on photophosphorylation[26,28,29,33,34]. However, the addition of compounds that dissipate both components of the high energy state, quite effectively prevent ATP synthesis in chromatophores[26].

The quenching is certainly not a measure of the capacity to synthesize ATP, since appreciable quenching occurs in the presence of valinomycin and nigericin or NH_4Cl under conditions that abolish phosphorylation. However, compounds such as nigericin, ammonium salts, and CCCP, which prevent the uptake of protons, effectively reverse the fluorescence quenching (Table I). Furthermore, the addition of valinomycin at concentrations which collapse the membrane potential still permits extensive quenching. This can be explained in the context of H^+ uptake, since valinomycin not only increases the initial rate of proton uptake, but also stimulates the efflux of protons in the dark[26]. This is then manifest in a faster reversal of the quenched fluorescence in the dark, followed by a much more rapid quenching during subsequent illuminations. The kinetics of quenching in chromatophores are then similar to those observed in chloroplasts.

The exact mechanism of the diaminodurene-catalyzed quenching of fluorescence is not known, nor is the connection to proton uptake readily apparent. It is possible that the protons accumulated may protonate certain molecular species and make them more efficient quenchers. The long times necessary for the production of the fully quenched state, however, would seem to imply that mechanisms such as the "membrane Bohr effect"[42] would not be of primary importance. Perhaps the protonated form of diaminodurene is a specific quencher of bacteriochlorophyll fluorescence.

The use of the diaminodurene-catalyzed light-induced quenching of bacteriochlorophyll fluorescence as an "intrinsic" probe to study proton uptake may be of value. It is more sensitive and can yield a much greater signal to noise ratio than glass electrode measurements, something that is quite significant when using *R. spheroides* chromatophores. Of course, the importance of the method will be increased if a more quantitative relationship between quenching and proton uptake can be established. Understanding the mechanism of this fluorescence quenching may also lead to greater knowledge about the relationship between the primary photochemistry and the high energy state, a problem which is pursued further in the following article[43].

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