Protein phosphorylation as a control for excitation energy transfer in *Rhodospirillum rubrum*

Nigel G. Holmes and John F. Allen

Department of Plant Sciences, University of Leeds, Leeds LS2 9JT, England

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Changes in cooperativity of the light-harvesting system of *Rhodospirillum rubrum* were monitored in whole cells by measurements of in vivo bacteriochlorophyll fluorescence yield transients. Under non-cooperative conditions a polypeptide of 10.5 kDa (provisionally identified as B880-β) was specifically phosphorylated; under cooperative conditions a polypeptide of 13 kDa (provisionally identified as B880-α) was specifically phosphorylated. These results are discussed in relation to the regulation of excitation energy distribution in purple photosynthetic bacteria and other photosynthetic organisms.

Photosynthesis *Purple bacteria* *Light-harvesting complex* *Photosynthetic unit* *Protein phosphorylation* *Excitation transfer* *Bacteriochlorophyll fluorescence*

1. INTRODUCTION

The photosynthetic apparatus of purple non-sulphur bacteria is considered to be organised such that light energy absorbed into one photosynthetic unit can be transferred to a neighbouring unit – the 'lake model' [1–4]. Protein phosphorylation is a widespread mechanism for the control of distribution of excitation energy in both prokaryotes and eukaryotes [5–9]. Recent observations in *Rhodospirillum rubrum* by Loach and coworkers [10,11] have indicated that, under some conditions, energy transfer between photosynthetic units becomes inhibited. This they describe as a change from a cooperative to a non-cooperative state. They correlate these changes with phosphorylation of the B880-α subunit of the light-harvesting pigment-protein complex under cooperative conditions. We have extended these observations, by using ^{32}P labelling and fluorescence studies, and discuss the data within the framework of a general model for the control of excitation energy distribution in photosynthetic systems [5].

Abbreviations: F_0, fluorescence yield when all photochemical traps are open; F_m, fluorescence yield when all photochemical traps are closed; F_t, fluorescence yield at time, t; LHC-II, light-harvesting chlorophyll *a/b* pigment-protein complex 1

2. MATERIALS AND METHODS

Cells of *R. rubrum* were grown in Sistrom's medium [12] but with the phosphate concentration reduced to 1 mM. Illumination was provided by two banks of tungsten filament lights, each of three 60 W bulbs, set 1 m apart. For labelling experiments, cells were grown, in a bijou bottle, holding about 7 ml, from an inoculum of 0.5 ml culture, with 1 mCi $^{32}P_i$. When the cells had reached late logarithmic phase, 4 × 1.5 ml aliquots were taken, pelleted in an Eppendorf centrifuge and resuspended under the conditions in [10,11] to give cooperative and non-cooperative cells. These conditions were either 20 mM KP_i, pH 6.8, incubated for 2 h in the dark (non-cooperative) or 20 mM KP_i, 20 mM $MgSO_4$, pH 6.8, incubated for 2 h in the light (cooperative). An additional 250 μCi $^{32}P_i$ was added to each of the incubations

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to ensure retention of labelled phosphate. The cells were precipitated with 5% trichloroacetic acid to terminate the reaction and were pelleted, washed with 10 mM Tris, pH 8.0, and extracted exhaustively (7 times) with 80% acetone; between each extraction the samples were left at −20°C. Dissociating buffer [9] was added and the samples incubated at 60°C for 30 min. Samples were treated with RNase, and with pronase where indicated, as in [13], then run on an 11.5–16.5% acrylamide gel with 5% stacking gel [19]. The gel was subsequently extracted with 16% trichloroacetic acid for 45 min and 5% trichloroacetic acid for 12 h at room temperature to remove polyphosphates and any residual nucleic acids which would otherwise obscure the autoradiograph [14]. The gels were stained, destained and autoradiographed as in [9].

Fluorescence was measured at right angles to the excitation beam in a fluorimeter constructed in this department. Exciting light was provided by a Volpi quartz-halogen 250 W light source (CUEL, Kenilworth, England) and blue light selected by a combination of a Corning 4-96 and a Calflex C filter; the beam was transmitted to the cuvette (10 mm square) via a fibre-optic light guide (Schoelly, FRG) and electronic shutter (Uniblitz, Vincent Associates, Rochester, NY). Fluorescence was detected by a Hansatech type FDP photodiode with associated amplifier (Hansatech, Kings Lynn, Norfolk) protected by a Wratten 88A filter with a Schott 891 nm interference filter (half-width 55 nm). The signal from the amplifier was recorded by a Farnell DTS 12P digital storage oscilloscope and X-Y recorder (Farnell Instruments, Wetherby, England). The opening time of the shutter was about 1 ms.

Ribonuclease A and pronase E (protease type XIV) were from Sigma. Other chemicals were from Sigma or BDH and were Analar or equivalent. $^{32}P_i$ was from Amersham International, England.

3. RESULTS

The kinetics of the transient rise of chlorophyll fluorescence yield can be used to estimate the extent to which energy is able to pass between photosynthetic units. This rise from F_0 to F_m occurs on a millisecond time scale and has been used to monitor cooperativity of photosynthetic units in both algae and bacteria [15–17]. As the probability of transfer between units increases, the curve of the transient becomes more sigmoidal in appearance – this can be seen more clearly in a semi-logarithmic plot [17]. Fig.1a compares the fluorescence yield transients in samples incubated under the conditions described above. The shape of the transient in cells incubated in the presence of Mg^{2+} in the light shows a greater degree of sigmoidicity than those transients from cells incubated in the dark without Mg^{2+} – this can be seen in the semi-logarithmic plot in fig.2. These measurements are consistent with the light-harvesting system being in a non-cooperative state in cells incubated in the dark without Mg^{2+}, and

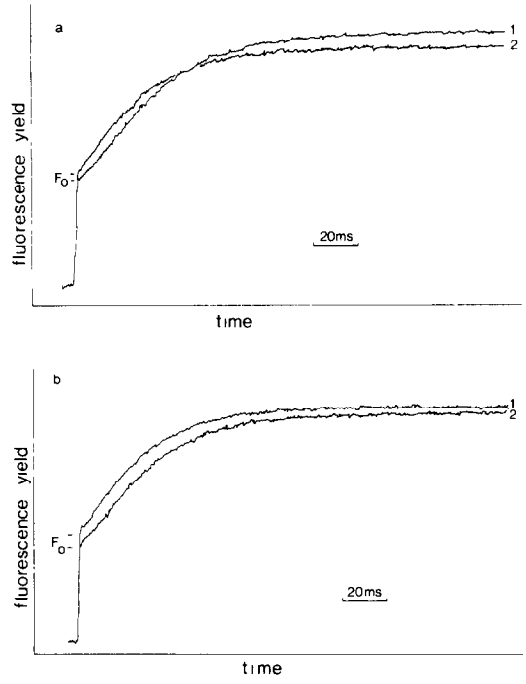

Fig.1. Fluorescence yield transients of cells of *R. rubrum*. Cells were incubated either in the dark in 20 mM KP_i, pH 6.8, or in the light in 20 mM KP_i, 20 mM $MgSO_4$, pH 6.8, as described in the text, and diluted 10 times for the measurement of fluorescence. (a) Cells suspended to A = 0.58 at 880 nm. Curves: 1, cells incubated in light with 20 mM $MgSO_4$; 2, cells incubated in dark without 20 mM $MgSO_4$. (b) Cells suspended to A = 0.58 at 880 nm. Curves: 1, cells incubated in dark without 20 mM $MgSO_4$; 2, same sample but with addition of 20 mM $MgSO_4$ and left in the dark for 2 min.

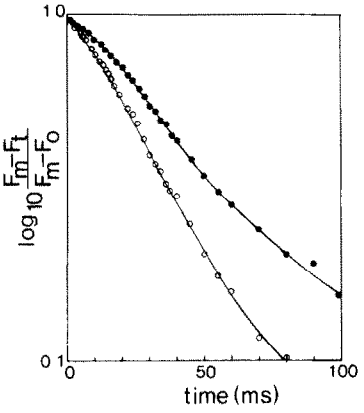

Fig.2. Analysis of fluorescence yield transients for cells incubated in light with 20 mM $MgSO_4$ (●) and for cells incubated in dark without 20 mM $MgSO_4$ (○). F_m, maximum fluorescence measured over a period of 5 s; F_t, fluorescence measured at time t after the shutter was fully open.

the light-harvesting system being in a cooperative state in cells incubated in the light with Mg^{2+}, in agreement with the suggestion of Loach et al. [10,11]. Beyond about 50 ms the semi-logarithmic plot of each transient shows a further departure from linearity, possibly because the *o*-phenanthroline block works imperfectly and permits additional turnover of reaction centres on a longer time scale. We have also noted a lowering of F_0 in the presence of Mg^{2+}. This can be seen in measurements of the type shown above but can be shown particularly clearly on addition of Mg^{2+} to cells incubated in the dark without Mg^{2+}. In fig.1b are shown fluorescence transients from cells incubated in the dark without Mg^{2+} before and after addition of 20 mM $MgSO_4$. Addition of Mg^{2+} has two clear effects on the kinetics of the fluorescence yield: F_0 is lowered and there is a change in the shape of the transient. The fall in F_0 is of the order of 7–10%. Analysis of the shape of the transient indicates that there is again an increase in the sigmoidicity of the curve and that it falls between the two curves shown in fig.2. F_m also showed a decrease of 10%, but since this was only measured after several seconds it may represent a response to other changes such as in electron transport. Addition of a 0.9% sucrose solution, isotonic with 20 mM $MgSO_4$, had no significant effect on the fluorescence yield, indicating that these changes are not due to osmotic effects (not shown).

Cells were also incubated under conditions to give different degrees of cooperativity, as above, in the presence of $^{32}P_i$ and were analysed by SDS-PAGE and autoradiography. Fig.3 shows that several pronase-sensitive bands are phosphorylated of 51, 20.5, 17, 13 and 10.5 kDa. The strongly labelled bands running near the front of the gel are not pronase-sensitive and so are presumably not protein. Of these labelled polypeptides, those of 17 and 13 kDa were phosphorylated only in the cooperative cells while the 10.5 kDa polypeptide was phosphorylated strongly in non-cooperative cells but only weakly in cooperative cells. In contrast, the labelled bands of 51 and 20.5 kDa were phosphorylated to a similar extent under both incubation conditions. The 13 and 10.5 kDa bands appearing on the autoradiograph correspond with bands staining with Coomassie blue on the gel, although the stained band at 13 kDa will also contain RNase added during preparation of the sample. Phosphorylation of polypeptides of 13 and 10.5 kDa has been reported previously for cell-free

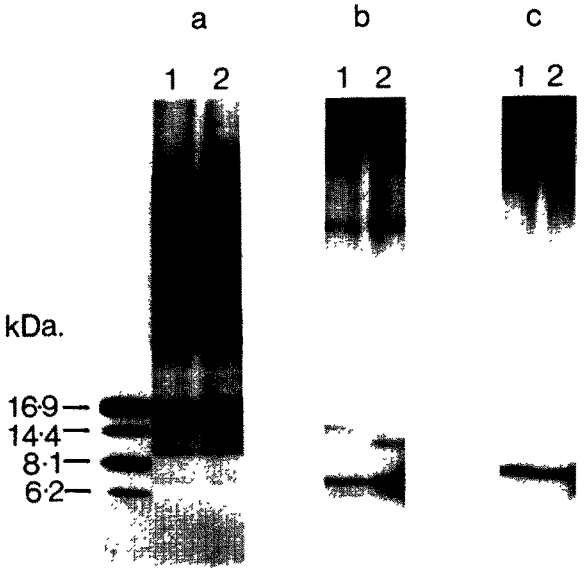

Fig.3. Molecular mass markers (LKB low molecular mass markers, myoglobin fragments) are shown in the track to the far left then (a) gel stained with Coomassie blue, (b) autoradiograph of (a,c) gel of pronase-treated samples showing: 1, cells incubated in light with 20 mM $MgSO_4$; 2, cells incubated in dark without 20 mM $MgSO_4$.

preparations of *R. rubrum*, but was not correlated with any functional measurements [18]; no labelled bands of 51 or 20.5 kDa were reported by these authors.

We identify the 13 kDa polypeptide with B880-α and the 10.5 kDa polypeptide with B880-β. The 13 kDa polypeptide is then the same polypeptide as the organic-soluble polypeptide reported in [10] to be labelled in cooperative cells. The 10.5 kDa polypeptide, phosphorylated under non-cooperative conditions, would not have been seen by those authors because of its insolubility in organic solvents [19]. The identity of the weakly phosphorylated 17 kDa component is unknown.

4. DISCUSSION

As pointed out in [11], if the phosphorylation site for B880-α is presumed to be either a serine or a threonine residue (as in higher plants [7]), then, from the sequence of the polypeptide [20], the site has to be on the periplasmic surface of the membrane. There is a precedent for such a phosphorylation of an amino acid residue situated in the periplasmic space in gram-negative bacteria. In *Escherichia coli* an arginine-ornithine binding protein situated outside the membrane, and involved in membrane translocation, is phosphorylated (using ATP as the phosphate donor), probably at a carboxyl residue [21], using ATP as a phosphate donor. There are several serine and threonine residues in B880-β [21] which offer potential phosphorylation sites, on both the cytoplasmic and periplasmic sides of the membrane. In a cell-free system of *R. rubrum* [18], a polypeptide of 11 kDa was labelled (corresponding to our 10.5 kDa polypeptide) with a 13 kDa polypeptide being less strongly labelled; when the membranes were separated from a soluble fraction only the 11 kDa polypeptide was phosphorylated. The conditions for incubation (20 mM Tris-HCl, 5 mM $MgCl_2$, pH 8.0, for 15 min) [18] were different from those used here and may not have favoured phosphorylation of the 13 kDa component.

Our measurements of the fluorescence yield changes (fig.1a) confirm the cooperativity changes suggested [11] on the basis of a different technique for estimating energy transfer between units. In addition, we observe (fig.1b) that addition of Mg^{2+} to cells in a non-cooperative state partly mimics the effect of incubation in the light in the presence of Mg^{2+}. This suggests that the effect is dependent on Mg^{2+} rather than on illumination.

Whatever the mechanism of the change in cooperativity, it must involve some relative movement of the light-harvesting components of neighbouring photosynthetic units. Some sort of lateral movement of the complexes would seem the most likely possibility, and would then be analogous to LHC-II movement in chloroplasts, although other mechanisms could be envisaged since energy transfer depends on a number of other parameters [4] such as orientation of chromophores.

It has been reported that on incorporation of lipid into chromatophores there is a change in the extent of energy transfer between pigment-protein complexes in *Rhodopseudomonas sphaeroides* [23]. In proteoliposomes made with light-harvesting II complexes from *Rps. palustris* as little as 2 mM Mg^{2+} was seen to cause lateral aggregation of the complexes [24]. It is therefore clear that these pigment-protein complexes are mobile in the membrane and are responsive to the ionic environment.

Elsewhere we have argued [5] that phosphorylation is a mechanism for inducing aggregation and de-aggregation of photosynthetic membrane proteins through modification of charge on exposed regions of the polypeptide chain. We also propose [5] that this is a general regulatory mechanism found in both eukaryotes and prokaryotes. The induction of non-cooperative conditions by phosphorylation of B880-β fits well with this model, with mutual electrostatic repulsion between the neighbouring units forcing their dissociation. It is not yet clear how the phosphorylation of B880-α could give rise to increased cooperativity. If the phosphorylated residue is on the periplasmic side of the membrane and the phosphorylated residue of B880-β is on the cytoplasmic side, then phosphorylation of either subunit may reinforce the dephosphorylation of the other.

The change in magnitude of F_0 on addition of Mg^{2+} (fig.1b) is unexpected; it may be that Mg^{2+} changes one or more of the rate constants on which fluorescence yield depends [1,3,4]. Alternatively, if some of the light-harvesting complex exists free in the membrane unconnected to the reaction centres

and with an enhanced fluorescence yield (as in the case of isolated light-harvesting complex [25]), then the effect of Mg^{2+} could involve not only bringing dissociated photosynthetic units together, but also bringing free light-harvesting complexes into functional contact with photosynthetic units. This could quench fluorescence yield, even with all the photochemical traps open.

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