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Protein phosphorylation in chromatophores from *Rhodospirillum rubrum*

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On incubation with [γ-^{32}P]ATP, a number of polypeptides of chromatophore and soluble fractions of *Rhodospirillum rubrum* are phosphorylated, including polypeptides of 70, 62, 61, 57, 49, 30, 22, 17, 13 and 10 kDa. Phosphorylation is stimulated by illumination and by addition of the oxidant potassium ferricyanide but is inhibited in the presence of the reductant, sodium dithionite. The electron-transport inhibitor, dibromomethylisopropylbenzoquinone (DBMIB), also inhibits light-stimulated protein phosphorylation. Phosphorylation of the 10 kDa polypeptide requires the readdition of a supernatant fraction to the membrane fraction. The data are compatible with a model we propose in which phosphorylation of B880 subunits is regulated by a component of the electron-transport chain in the vicinity of the ubiquinone pool.

Introduction

Protein phosphorylation is now well established as a mechanism for regulating enzyme activities and protein interactions in eukaryotes [1]. Evidence is accumulating for its significance in prokaryotes [2–8]. There are several reports of the presence of phosphorylated proteins in purple photosynthetic bacteria [5–8], and the phosphorylations of two polypeptides, of 13 and 10.5 kDa, have been correlated with changes in excitation energy distribution in the light-harvesting system of *Rhodospirillum rubrum* [5,6]. We have suggested that these polypeptides may be identified as B880-α and B880-β [6]. We have discussed elsewhere [9] how the regulation of excitation energy transfer in purple photosynthetic bacteria, in cyanobacteria and in higher plants can be considered within one, unifying, framework. In cyanobacteria the substrates for this phosphorylation may be polypeptides of the phycobilisome and of Photosystem II [10,11] and in chloroplasts the substrate for phosphorylation is known to be the light-harvesting pigment-protein complex, LHC-II [12–14].

Only one type of light-harvesting complex has been purified from *R. rubrum* and the photosynthetic unit of *R. rubrum* probably consists therefore of just two species of pigment-protein complex, the reaction centre together with its associated, bound, light-harvesting complexes [15]. The pigments in the light-harvesting complexes are bound, non-covalently, to two apoproteins designated B880-α and B880-β [15]. These photosynthetic units are thought to be associated together in such a way that absorbed excitation energy can pass freely between them – the 'Lake Model'

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; LHC-II, light-harvesting chlorophyll *a*/*b* pigment-protein complex of Photosystem II; UQ, ubiquinone; UQH_2, ubiquinol; PAGE, polyacrylamide gel electrophoresis.

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[17–19]. However, there is evidence that it is possible for the photosynthetic units to become functionally decoupled [5,6] and there is a correlation between the degree of coupling of photosynthetic units and phosphorylation of polypeptides identified as being subunits of the light-harvesting complex; phosphorylation of the 13 kDa polypeptide correlates with increased cooperativity between photosynthetic units, while phosphorylation of the 10.5 kDa polypeptide correlates with decreased cooperativity [5,6] between photosynthetic units.

The photosynthetic apparatus of *R. rubrum* is situated on the intracytoplasmic membrane [20]. Vesicles formed from this membrane can be isolated as chromatophores. Holuigue et al., using [γ-^{32}P]ATP in a cell-free system, demonstrated the phosphorylation of a number of polypeptides, including two of 13 kDa and 11 kDa (by SDS-PAGE) appropriate for the apoproteins of B880 [8]. We have previously reported protein phosphorylation in whole cells of *R. rubrum* [6]. We report here independent evidence indicating that chromatophores of *R. rubrum* contain protein kinase activity which results in a phosphorylation of chromatophore polypeptides. We also present evidence that the phosphorylation is under redox control, with phosphorylation being stimulated under oxidising conditions.

Materials and Methods

Cells of *R. rubrum* were grown in Sistrom's medium [21], but with the phosphate concentration reduced to 1 mM. Illumination was provided by 2×60 W and 2×150 W tungsten filament lights at approx. 0.5 m from the cultures; the cultures were in 0.5 litre flat-sided bottles. The cells, in mid-log phase, were harvested by centrifugation for 5 min at $16000 \times g$ and washed in 20 mM potassium phosphate/20 mM $MgSO_4$ (pH 6.8). The cells were resuspended in 20 ml of the same buffer and broken in a French Pressure Cell (Aminco, Silver Springs, MD) at 110 MPa in the presence of ribonuclease *a* and deoxyribonuclease 1. Chromatophores were separated from cell debris by centrifugation for 15 min at $33000 \times g$ and were collected by centrifugation for 1.5 h at 144000 *g*. Chromatophores were re-suspended in the minimum volume of 20 mM potassium phosphate/20 mM $MgSO_4$ (pH 6.8). Bacteriochlorophyll was estimated from the in vivo absorption coefficient given in Ref. 22.

Chromatophores, containing 20 μg bacteriochlorophyll, were incubated in a total volume of 0.5 ml in 20 mM KP_i/20 mM $MgSO_4$ (pH 6.8) in an Eppendorf-type centrifuge tube (Sarstedt, Nümbrecht, F.R.G.) under the conditions given. Venturicidin at 2 $\mu g \cdot ml^{-1}$ was routinely included to inhibit the ATP synthase/ATPase activities of the coupling factor. After 15 min dark incubation, the reaction was started by the addition of ATP to a final concentration of 400 μM and containing a total of 30 μCi of [γ-^{32}P]ATP (New England Nuclear Research Products, Du Pont Ltd., Stevenage, U.K.). The reaction was stopped by addition of trichloroacetic acid to 5%. The samples were pelleted, washed with 20 mM Tris (pH 8.0) and extracted exhaustively (at least four times) with cold 80% acetone; between each extraction the samples were left at $-20\,^{\circ}$C. Dissociating buffer [6] was added and the samples incubated at $60\,^{\circ}$C for 30 min. SDS-PAGE was performed on an 11.5–16.5% acrylamide gel with 5% stacking gel [6]. The gels were stained, destained and autoradiographed as in [6].

Chemicals were from Sigma or BDH and were AnalaR or equivalent.

Results

Chromatophores from *R. rubrum* were incubated for 15 min in the presence of [γ-^{32}P]ATP and the polypeptides were separated by SDS-PAGE. Fig. 1 shows an autoradiograph from the resulting gel. Although phosphorylation takes place both in the light and the dark, comparison of tracks (a)1 (incubated in the light) and (a)2 (in the dark) shows that phosphorylation of some of the polypeptides is stimulated in the light. This is particularly evident in the part of the gel corresponding to polypeptides of low molecular weight where a polypeptide of 13 kDa is clearly labelled more strongly in the light than in the dark. In an earlier publication [23], we have reported that the band seen on autoradiographs in this low-molecular-weight region, in the light, corresponds to two polypeptides. On the basis of further experiments, it is now clear that only one band at 13 kDa is

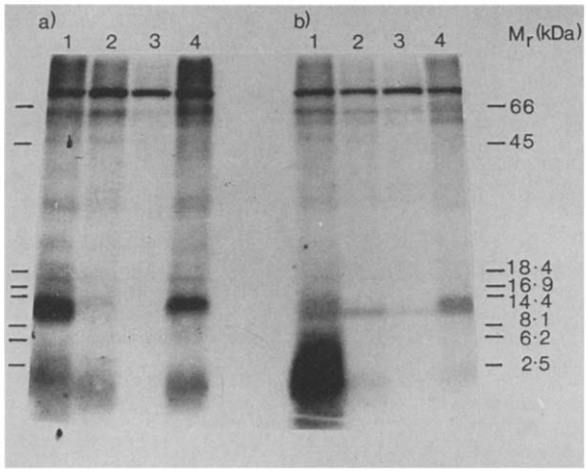

Fig. 1. Phosphorylation of chromatophore polypeptides. Autoradiograph of SDS-PAGE of chromatophore sample incubated as in Materials and Methods. Samples were incubated as follows: (1) light; (2) dark; (3) dark with 5 mM sodium dithionite; (4) dark with 2 mM potassium ferricyanide. Tracks (a)1–4 were incubated in the absence and (b)1–4 in the presence of added supernatant.

being labelled (Fig. 1). The phosphorylation of polypeptides of 29, 22 and 17 kDa is also stimulated in the light. Other polypeptides present, of 70, 61, 57 and 49 kDa are phosphorylated in a reaction that shows no stimulation by light. Track (a)4 shows that the polypeptides that show light-stimulated phosphorylation can also be phosphorylated in the dark in the presence of 2 mM potassium ferricyanide. This observation suggests that the phosphorylation of these components is under redox control, and that the kinase responsible is activated under oxidising conditions. In the presence of the strong reductant, sodium dithionite (5 mM), in the dark (track (a)3) only the polypeptide of 70 kDa is phosphorylated. It may be that this is another indication of redox control on the kinase(s) responsible, although it is also possible that dithionite is such a strong reducing agent that it may reduce the kinase(s) directly and irreversibly inactivate them.

We have explored further the conditions required for phosphorylation of those polypeptides which show a light stimulation of phosphorylation. The activation of the protein kinase responsible for phosphorylating these bands by ferricyanide, in the dark, suggests it may be regulated by the redox poise of an electron-transport component. This would indicate similarities to the LHC-II protein kinase of higher plants [12–14] and probably also to the protein kinase which phosphorylates a component of the phycobilisome fraction in cyanobacteria [10,11]. An important difference to note when comparing the activation of this kinase with the kinase phosphorylating LHC-II in higher plants is that the LHC-II kinase is activated by reduction of the quinone pool, whereas the kinase identified here in *R. rubrum* would be stimulated by oxidation of the quinone pool. To examine this possibility, we have looked at the effect of electron-transport inhibitors on light-stimulated protein phosphorylation.

DBMIB and antimycin a both inhibit electron flow in the region of the cytochrome b-c_1 complex. Their precise sites of action, however, are believed to be different: DBMIB blocks reoxidation of UQH_2 from the quinone pool at the Q_c site, whereas antimycin a inhibits electron flow near to the Q_z site [24]. In Fig. 2, the effects of DBMIB and of antimycin a on light-activated protein phosphorylation are shown. These results are compared with those observed in an uninhibited control incubated in the light. When DBMIB (at 10 μM) is added to chromatophores incubated in the light in the presence of [γ-^{32}P]ATP, an almost total inhibition of labelling is observed (Fig. 2). By contrast, antimycin a (2 μM) does not have such a marked effect, but nevertheless a difference can be seen in the labelling of the 13 kDa polypeptide in the presence and absence of antimycin a (Fig. 2). Given that the evidence cited above indicates that the kinase is activated under oxidising conditions, this evidence indicates a site of regulation for the kinase between the reaction centre and the cytochrome b-c_1 complex. Our results are compatible with the idea that the component responsible in *R. rubrum* is the ubiquinone pool. We believe that our failure to see total inhibition of phosphorylation in the presence of antimycin a is, in fact, due to an incomplete inhibition of electron flow by this inhibitor under these conditions.

Fig. 1, tracks (b)1 to (b)4, show membranes incubated under the same conditions as tracks a(1) to (a)4, but in the presence of the 144 000 $\times$ g supernatant. The incubation medium therefore includes the soluble fractions from the cytosol and

from the periplasm. A clear difference between these samples and the samples incubated in the absence of supernatant is that in the presence of supernatant a 10 kDa polypeptide is phosphorylated, irrespective of the other conditions (Fig. 1, tracks (b)1–4). This component is phosphorylated even in the dark in the presence of 5 mM sodium dithionite (Fig. 1, (b)3), in contrast to light-stimulated protein phosphorylation, which is virtually eliminated by the presence of dithionite.

Fig. 3 shows a comparison of chromatophores incubated without supernatant (track 1) and with supernatant (track 2) together with supernatant incubated alone (track 3); all incubations being in the dark. Phosphorylation of the 10 kDa polypeptide is seen only in track 2, in the presence of both fractions. An additional supernatant polypeptide of 62 kDa is seen to be phosphorylated in both tracks 2 and 3; this is apparently phosphorylated by a different, soluble kinase activity, indicating that there are at least two different kinase activities in this species.

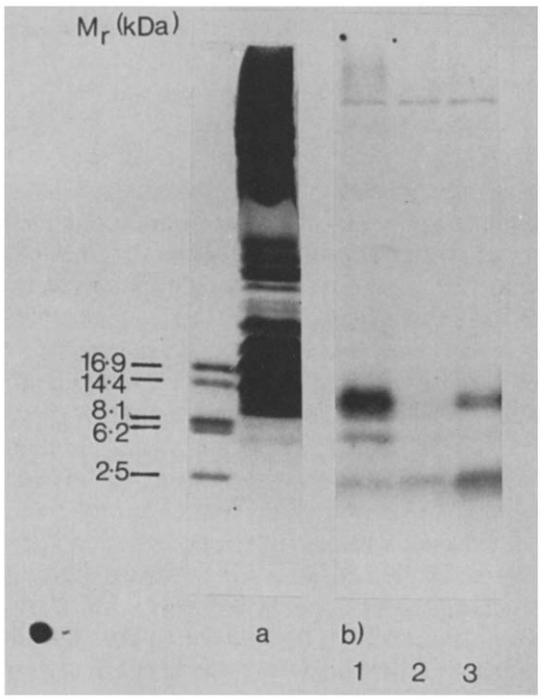

Fig. 2. Effect of illumination on phosphorylation of chromatophore polypeptides. Chromatophores were incubated as in Fig. 1. Track (a) shows Coomassie blue staining bands. Tracks (b) are autoradiographs. Incubation conditions were: (b) 1, light, no addition, (b) 2, light with 10 μM DBMIB, (b) 3, light with 2 μM antimycin a.

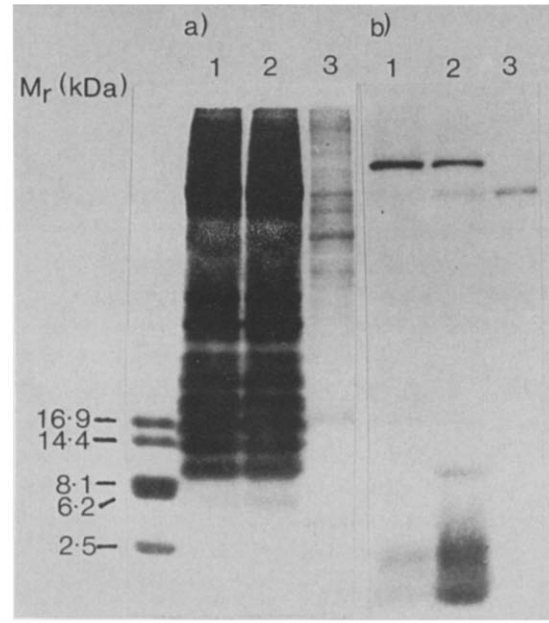

Fig. 3. Effect of reconstitution with soluble fraction on phosphorylation of chromatophore polypeptides. Chromatophores were incubated as in Materials and Methods. Incubations were in the dark and as follows: (1) chromatophores alone; (2) chromatophores with supernatant; (3) supernatant alone. (a) Gel stained with Coomassie blue; (b) autoradiograph.

Discussion

In these experiments we have identified a number of phosphorylated bands labelled in *R. rubrum*: several in chromatophores with 70, 61, 57, 49, 30, 22, 17 and 13 kDa. one in the soluble fraction of 62 kDa and one, of 10 kDa, which requires both the chromatophore and the soluble fractions. Table I shows a comparison of the phosphorylated polypeptides reported from photosynthetic purple bacteria in a number of studies. It is interesting to note that many of the bands reported from in vivo labelling of *R. rubrum* have their counterparts in these chromatophore labelling experiments, implying that they are either membrane-bound or are located in the periplasmic space and are therefore trapped within the chromatophore lumen. The work of Holuigue et al. [8] using a similar in vitro system of *R. rubrum* has reported major phos-

TABLE I

SUMMARY OF REPORTED PHOSPHORYLATED POLYPEPTIDES IN PHOTOSYNTHETIC PURPLE BACTERIA

Values are masses in kDA of the phosphopolypeptides reported. In column 2, (s) indicates found in the soluble fraction, (m) found in the membrane fraction.

Rhodospirillum rubrum [b]			*Rhodomicrobium vannielli* [c]		*Rhodobacter sphaeroides*
in vitro [a]	in vitro [8]	in vivo [6]	in vitro [29]	in vivo [29]	in vivo [7]
	90 (s)				
	86 (m)			88	
70					
	64 (s)			66	
61			60		59
57			57	55	
49		51	50		
					45
			42		
30	31 (s)		29		
22		21			
17		17	15		16
13	13	13		13	12
10 [d]	11 (m) [3]	11			

[a] This study.

[b] In addition, Loach et al. [5] report the phosphorylation of B880-α (apparent mass 13 kDa [15], real molecular mass 6 kDa [26]) under defined incubation conditions.

[c] Variations in the polypeptides phosphorylated with age and with stage of differentiation of cells. A number of less strongly identified polypeptides were also noted.

[d] Possibly found in the supernatant. There is also a band of 62 kDa in the supernatant.

phorylated polypeptides as in Table I. These authors do not specify the illumination conditions in their study. They suggest that the 13 kDa phosphopolypeptide may be a component of B880, in line with the suggestions of Loach and co-workers [5] who have reported the phosphorylation of B880-α. From Table I it can be seen that a number of phosphopolypeptides are common to several or all of the studies. Bearing in mind differences in experimental procedure and uncertainties in the estimation of polypeptide molecular masses by SDS-PAGE (particularly in the case of membrane proteins), it is nevertheless possible to identify phosphopolypeptides of approx. 60, 57, 50, 30, 16, 13 and 11 kDa, each of which is common to a number of the studies.

In the chromatophore fraction, the kinase activity is stimulated both by illumination and by addition of 2 mM potassium ferricyanide. It is inhibited by addition of 5 mM sodium dithionite. This implies that the ambient redox potential is a controlling factor on kinase activity, and opens up the possibility that the kinase activity is regulated by the redox poise of a component of the photosynthetic electron-transport chain, in a way similar to the regulation of the phosphorylation of LHC-II in higher plants [12–14]. By analogy with the higher plant system, we would predict that the site for regulation of the kinase would be at, or near to, the quinone pool – ubiquinone in the case of *R. rubrum* [25]. The highly oxidising conditions generated by 2 mM ferricyanide ($E_m = +430$ mV) [26] would be expected to maintain the ubiquinone pool oxidised, whereas the strong reductant sodium dithionite would be expected to reduce totally the ubiquinone pool. The experiment with DBMIB gives information on the location of the regulatory site for the kinase activity. If the kinase is activated by oxidation of a component of the electron-transport chain, the inhibition of light-stimulated phosphorylation by DBMIB would suggest that the site of regulation is in the electron-transport chain between the reaction centre and the cytochrome b-c_1 complex. This observation is compatible with

a model in which ubiquinone is the site of regulation for the kinase. It should be noted, however, that there is evidence from studies of the phosphorylation of LHC-II in pea chloroplasts that DBMIB, at concentrations sufficient to act as a Photosystem II electron acceptor, may also act directly to inactivate a protein kinase [27]. At present we cannot eliminate the possibility that this is occurring in *R. rubrum*.

In chromatophores of *Rhodobacter sphaeroides* we have previously observed an inhibition of light-stimulated phosphorylation by addition of 0.5 mM duroquinol [7]. Duroquinol is believed to donate electrons directly to the quinone pool [30] and will therefore inhibit any oxidation of ubiquinone. This suggests that similar mechanisms for regulation of kinase activity are operating in both *R. sphaeroides* and *R. rubrum*.

The 10 kDa phosphopolypeptide requires a soluble factor in addition to a membrane fraction for its phosphorylation. This polypeptide may be a membrane-bound or a periplasmic polypeptide requiring a regulatory component, or possibly the kinase itself, from the supernatant fraction. The supernatant fraction clearly contains kinase activity, since we observe phosphorylation of a polypeptide of 62 kDa present in the supernatant fraction. Alternatively, the 10 kDa phosphopolypeptide may be in the soluble fraction and the component necessary for its phosphorylation in the membrane fraction.

We suggest that the 13 kDa phosphopolypeptide can be identified with the 13 kDa phosphopolypeptide which is seen in whole cells, and the phosphorylation of which correlates with an increase in functional connectivity of the photosynthetic units [6]. We conclude from the above evidence that phosphorylation of the 13 kDa polypeptide is activated by oxidation of an electron-transport component at or near the ubiquinone pool, and is inhibited by its reduction.

If the function of the phosphorylation-linked changes in excitation energy transfer is to compensate for conditions, either metabolic or environmental, that lead to a non-optimal rate of electron-flow through the reaction centre, then regulation by an electron-transport component would be a plausible mechanism of control. Moreover, the observed regulation of the phosphorylation of the 13 kDa polypeptide would fit well with such an idea: in whole cells, a low (light-induced) throughput of electrons to the acceptor side will lead to a net oxidation of the ubiquinone pool. This in turn would enhance the kinase activity and therefore increase the level of phosphorylation of the 13 kDa polypeptide, which will lead to more of the photosynthetic units becoming functionally connected and therefore to a more efficient utilisation of light energy and an increase in reaction centre turnover. Elsewhere [6] we have tentatively identified this polypeptide as B880-α; although the real molecular masses of B880-α and B880-β are known from the amino-acid sequence to be about 6 kDa [28], they behave on SDS-PAGE as if they were of 13 and 11 kDa, respectively.

We suggest that the 10 kDa polypeptide can be identified with the 11 kDa phosphopolypeptide reported in chromatophores [8] and the 10.5 kDa phosphopolypeptide reported in whole cells [6] of *R. rubrum* and the phosphorylation of which correlates with a decrease in functional connectivity of the photosynthetic units; we have tentatively identified this polypeptide as B880-β. The alternating pattern of phosphorylations of the 13 and 10.5 kDa polypeptides reported in whole cells of *R. rubrum* in Ref. 6 would suggest, from the above considerations, that the 10 kDa polypeptide should undergo phosphorylation/dephosphorylation under conditions complementary to the conditions for the 13 kDa phosphopolypeptide; it should therefore be phosphorylated when the ubiquinone pool is reduced and dephosphorylated when the ubiquinone pool is oxidised. Interpretation of the phosphorylation of this component is complicated in our in vitro system by its requirement for a soluble fraction, but we see no evidence for the inhibition of phosphorylation of this polypeptide in the presence of potassium ferricyanide. However, we do see phosphorylation of the 10 kDa polypeptide in the presence of the reductant sodium dithionite, in contrast to most of the other phosphopolypeptides. This is consistent with the phosphorylation of the 10 kDa polypeptide being activated under reducing conditions.

In Ref. 8 the authors report that a phosphopolypeptide of 13 kDa requires the presence of both the soluble and the membrane fractions based on fractionation of the total extract. This is similar

to the behaviour of the 10 kDa phosphopolypeptide that we report here based on fractionation followed by reconstitution. The methods used by Holuigue et al. and ourselves differ in several respects, most notably in the SDS-PAGE system used, and this may provide one possible explanation for the apparent anomaly in the molecular weights.

We conclude that the phosphorylation of a number of polypeptides of purple photosynthetic bacteria can be demonstrated in vitro by addition of [γ-^{32}P]ATP to a chromatophore fraction. This in vitro system opens up the possibility of further investigation of the kinase system and its regulation, using conditions better defined than in experiments using whole cells. The mechanism we propose for the regulation of excitation energy distribution in purple bacteria offers another example of the conservative nature of evolution at the molecular level. It also suggests that elucidating the details of this regulatory system in the purple photosynthetic bacteria may play a role in increasing our understanding of other membrane regulatory mechanisms which make use of covalent modifications of proteins.

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