Biochimica et Biophysica Acta, 1184 (1994) 28–36

BBAOBIO 43957

Protein phosphorylation in *Rhodospirillum rubrum*: purification and characterization of a water-soluble B873 protein kinase and a new component of the B873 complex, Ω, which can be phosphorylated

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(Received 4 August 1993)

Key words: Photosynthesis; Light-harvesting complex; Reaction center; Carotenoid; Protein kinase; Protein phosphorylation; (*R. rubrum*)

A water-soluble protein kinase (the B873 kinase), which can phosphorylate isolated B873 complexes, was purified from the water-soluble fraction of crude extracts from *Rhodospirillum rubrum* G9. The B873 kinase is monomeric with a molecular mass of approx. 20 kDa. Apart from B873 complexes, the enzyme is able to phosphorylate histones and ovalbumin. Although Mg^{2+} and Ca^{2+} are active as metal cofactors, a higher activity is observed in the presence of the transition metals Cu^{2+} and Mn^{2+}. The kinase activity is only weakly inhibited by ADP and AMP but no effect is observed upon the addition of cAMP. The B873 kinase is specific for serine residues. In vitro phosphorylation of the B873 complexes revealed that besides the α- and β-polypeptides, a third, new component, Ω, becomes labelled. This Ω component, which is not stained with Coomassie blue but by silver, is a polypeptide with a molecular mass of approx. 4 kDa.

Introduction

In both eucaryotes and procaryotes, protein phosphorylation is thought to play a central role in cellular regulation [1–4]. An important step in unravelling the complex events involved in many phosphorylation-dephosphorylation phenomena is the purification of the protein kinase(s) and their characterization. Whereas many eucaryotic protein kinases have now been studied [4,5], only a few procaryotic ones have been characterized [1].

In a preceding paper we have examined the phosphorylation of the B873 complexes of the carotenoidless mutant, *Rhodospirillum rubrum* G9 [6]. Phosphorylation of the α- and β-polypeptides of the complex, and of a new component, Ω, is catalysed by a water-soluble protein kinase activity with a pH optimum of approx. pH 7.5 and the enzyme is activated by transition metal ions such as Cu^{2+} and Mn^{2+}. In intact chromatophores, the B873 kinase activity seems to be modulated by the redox state of the membrane.

In this study we have isolated and characterized the B873 protein kinase present in the supernatant fraction obtained from cells of *R. rubrum* G9. Furthermore, we have characterized a third component of the complex, Ω, which is also phosphorylated by the B873 kinase. A preliminary report of this work has appeared previously [7].

Materials and Methods

Growth of bacteria and preparation of purified chromatophores was performed as described [6]. Chromatophores were suspended in 20 mM Tris-HCl pH 7.5 containing 5 mM $MgCl_2$ and then frozen in liquid nitrogen for storage at −80°C. The water-soluble supernatant fraction obtained after the first 100 000 × *g* centrifugation step was concentrated by ultrafiltration, and recentrifuged to remove contaminating membrane fragments. SDS-PAGE was performed according to Laemmli [8] and the B873 complexes were purified to homogeneity according to Ref. 9.

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Abbreviations: βOG, β-octylglucoside; DTT, dithiothreitol; TFA, trifluoracetic acid; PTH, phenylthiohydantoin; DBMIB, 2, 5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone.

SSDI 0005-2728(93)E0158-M

Purification of the B873 kinase activity

All of the following purification steps were performed at 4°C. Purification of the B873 kinase activity was achieved by affinity chromatography. 3 ml of the supernatant fraction was passed over a small (0.8 cm × 4 cm, total volume 2 ml)) ATP-C_6-agarose column (Sigma A-9264) pre-equilibrated with 20 mM Tris-HCl pH 7.5 containing 0.5 mM DTT and 1 mM $MgCl_2$. After recirculation of the eluate over the column for approx. 2 h at a flow rate of 1 ml/min, elution was performed using equilibration buffer containing 5 mM ATP and then 10 mM ATP. 400 μl fractions were collected, 16 μl samples were taken and analyzed by SDS-PAGE using a 12.5% SDS-gel followed by silver staining. In some cases the N_6-ATP agarose (Sigma A-2767) or ADP-Sepharose (Pharmacia) was used.

Fractions capable of phosphorylating B873 complexes were pooled and then applied to a Sephacryl 300 column (1 cm × 1.2 m, total volume 100 ml) pre-equilibrated with 50 mM Tris-HCl pH 7.5 containing 0.5 mM DTT and 1 mM $MgCl_2$. The flow rate of the column was approx. 5 ml/h. Fractions were collected every 15 min, and 200 μl aliquots lyophilized, dissolved in SDS sample buffer, and analyzed by SDS-PAGE. Fractions (500 μl aliquots) containing B873 kinase activity were pooled, then frozen in liquid nitrogen and stored at −70°C. 40 μl of the pooled fractions were subsequently employed for the measurement of phosphorylation activity.

Histone agarose chromatography was performed by passing the supernatant fraction (0.6 ml) repeatedly over a column (1 ml) of histone agarose (Sigma H-3889) which had been pre-equilibrated in 10 mM Tris-HCl pH 8.0 for 10 min. The column was washed with 500 ml 10 mM Tris-HCl/50 mM NaCl and then eluted with 10 mM Tris-HCl pH 8.0/250 mM NaCl. 0.5 ml fractions were collected and dialysed in an Eppendorf tube against 10 mM Tris-HCl pH 8.0 (2l) for 10 h. 16-μl aliquots were taken for analysis by SDS-PAGE.

Concentration of the B873 kinase for amino acid analysis and sequencing

The purified B873 kinase was found to bind irreversibly to Amicon ultrafiltration membranes. For protein chemical analysis the B873 kinase was concentrated by repeated extraction with 2-butanol as described [10]. Traces of 2-butanol present in the aqueous phase after extraction were removed by a single extraction with an equal volume of water-saturated ether. Control experiments with phycocyanin showed that the protein resides exclusively in the water phase after this procedure.

Determination of B873 kinase activity

In general the B873 kinase activity was determined by in vitro phosphorylation of a protein substrate (35 μg each of chromatophores, purified B873 complex, or purified histone) by [γ-^{32}P]ATP, followed by SDS-PAGE and autoradiography, essentially as described [6]. DBMIB (100 μM) and venturicidin (2 μg/ml) were added to the assay mix when whole chromatophores were employed as the protein substrate but were not necessary for the phosphorylation of purified B873 complexes or histone.

For some experiments phosphorylation was studied by a non-radioactive method for the detection of protein-bound phosphate [11] using the modification described below.

FPLC chromatography of purified B873 complexes

For FPLC separation of the isolated complexes, 200 μg of isolated complexes were dissolved in 50 mM NH_4HCO_3 pH 7.8, containing 1% (w/v) β-octylglucoside (βOG) in a total volume of 200 μl. The mixture was then loaded onto a PepRPC (Pharmacia) column equilibrated in 0.2% (v/v) trifluoracetic acid (TFA) in H_2O and eluted with CH_3CN/iPrOH (1:1 v/v) containing 0.2% (v/v) TFA by employing a continuous gradient. The absorption of the eluate was monitored at 280 nm and 0.2 ml fractions were collected. For analysis by SDS-PAGE, 100 μl of each fraction was neutralized with NH_4OH and evaporated to dryness under nitrogen. The residue was solubilized in sample buffer, containing 2% (w/v) SDS, heated at 100°C for 1.5 min, centrifuged at full speed in a bench top centrifuge for 5 min and applied to a 15% acrylamide gel containing 0.1% (w/v) SDS [8]. After separation the gel was stained using the silver stain kit from Bio-Rad.

Where appropriate, fractions were dried directly in pyrolyzed glass tubes, dissolved in 200 μl 6 N HCl + 0.001% phenol and hydrolyzed either at 110°C or 160°C for 24 h. The hydrolysate was dried under nitrogen, redissolved in 10 μl of 50 mM HCl, then 2 μl of the mixture was applied to an amino acid analyzer (Amino Quant, Hewlett-Packard). N-terminal sequencing was performed by Dr. H. Lahm, Hoffmann-La Roche. Samples were spotted onto a PVDF membrane and sequenced by Edman degradation using a pulsed liquid microsequencing apparatus (Applied Biosystems, USA) followed by automated HPLC-PTH-amino acid analysis (Applied Biosystems, USA).

Analysis of the phosphorylated amino acids

(a) Immunological determination of phosphotyrosine

Immunological detection of phosphotyrosine was obtained by a dot blot of phosphorylated samples against a monoclonal antibody specific for phosphotyrosine ([12]; ICN Biochemicals). 35 μg of phosphorylated protein was spotted onto a 0.45 μ nitrocellulose membrane (Schleicher and Schuell) and then blotted

with rabbit monoclonal antibody as described by Towbin et al. [13]. Detection was performed using a peroxidase-coupled anti-mouse antibody [13], and a positive signal appeared as a violet spot after 5–10 min. Haemocyanin (Sigma) or ovalbumin (Fluka), cross-linked to phosphotyrosine via glutaraldehyde were employed as positive controls. For the cross-linking reaction phosphotyrosine (100 nmol), glutaraldehyde (500 nmol) and protein (100 nmol) were dissolved in 2 ml 50 mM sodium phosphate buffer pH 7.0, and shaken for 2 h at room temperature. The reaction was stopped by adding 20 μl 1 M Tris-HCl pH 7.5 and dialyzed overnight. The labelled protein was used either directly or after lyophilization followed by resuspension in an appropriate volume of 20 mM Tris-HCl pH 8.0.

(b) Partial acid hydrolysis followed by high-voltage paper electrophoresis

^{32}P-labelled, purified B873 complexes (200 μg, approx. 16 nmol) or ^{32}P-labelled histone V-S (32 nmol), phosphorylated with 40 μl pooled B873 kinase, and pelleted with TCA, was washed with 20 mM Tris-HCl pH 8.0, dissolved in 50 μl 1% (w/v) SDS and diluted immediately with 450 μl 50 mM NH_4HCO_3 pH 7.8. The samples were incubated with 10 μg trypsin (Worthington) for 1 h followed by the addition of 1 μg of chymotrypsin (Worthington) for 4 h, then transferred to a 2 ml Reacti-Vial (Pierce) and lyophilized. 200 μl 6 M HCl (Pierce) was added to the vials and hydrolysis performed for 2 h at 105°C. After hydrolysis the acid was evaporated under a stream of nitrogen, and the hydrolyzed amino acids were dissolved in 10 μl of electrophoresis buffer (5% (v/v) acetic acid, 0.5% (v/v) pyridine, 94.5% (v/v) H_2O pH 3.5) and spotted onto 3MM paper pre-equilibrated in the same buffer. As a standard, 10 nmol each of phosphotyrosine, phosphoserine, and phosphothreonine (Sigma) were dissolved in 10% (w/v) SDS, then hydrolyzed as above and electrophoresed in the same system. Electrophoresis was conducted at 2000V for 80 min. in a Schleicher and Schuell high voltage electrophoresis apparatus. After separation, the paper was dried, stained with ninhydrin (0.1% (w/v) in 95% (v/v) ethanol) and the ^{32}P-labelled spots were detected by autoradiography.

(c) Non-radioactive detection of phosphorylated proteins

For some measurements, protein phosphorylation was measured by the malachite green detection method of Buss and Stull [11] with the following modifications. After TCA precipitation, the sample, containing 0.1–2 nmol phosphate, was dried in a glass tube at 80°C. 25 μl of ashing reagent (10% $MgNO_3 \cdot 6H_2O$ in 95% (v/v) ethanol) was added, the tube vortexed, and the content redried by gentle heating. The dried pellet was heated to a white residue, cooled, and then 150 μl 12 M HCl and 50 μl malachite green phosphate reagent [11] were added. Measurements of the optical absorption at 660 nm were made between 3–10 min after the addition of the phosphate reagent, during which time the colour remains stable. The cuvettes used for the phosphate determination were routinely washed and stored in 2 M HCl. Inorganic phosphate was employed for the determination of the standard curve.

Results

Purification of the B873 kinase

The B873 kinase was purified by affinity chromatography using N_6-ATP agarose. Fractions containing the

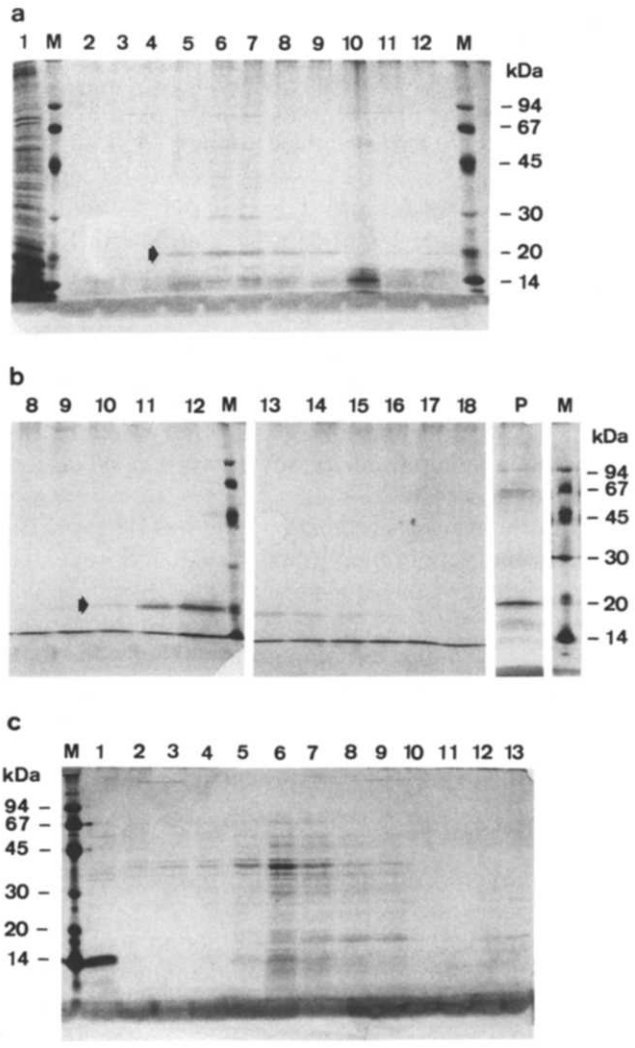

Fig. 1. SDS-PAGE followed by silver staining of fractions after chromatography of the water-soluble supernatant fraction obtained from a crude extract from *R. rubrum* G9 [6]. (a) Elution profile obtained after N_6-ATP agarose chromatography and elution with 5 mM ATP. Lane 1: starting material before chromatography, lanes 2–12: consecutive eluted fractions (0.5 ml each); lane M: the molecular weight marker. (b) Profile after Sephacryl 300 chromatography of the pooled fractions 5–9 obtained in (a). Lane P: pooled B873 kinase. The band corresponding to the B873 kinase is indicated. (c) Profile obtained after chromatography of the supernatant fraction using histone agarose. Lanes 1–13: eluted fractions; lane M: molecular weight marker.

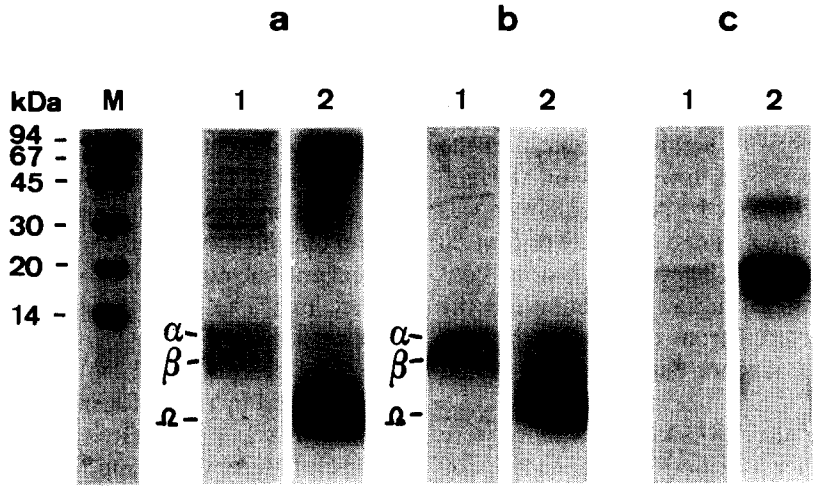

Fig. 2. Substrate specificity of the B873 kinase. Phosphorylation assays were performed using the substrates: (a) purified chromatophores; (b) B873 light-harvesting complexes; and (c) histone V-S; (35 μg of the target protein in each case), and analyzed by SDS-PAGE. The Coomassie-blue-stained SDS-PAGE profile (1) and the corresponding autoradiogram (2) are shown.

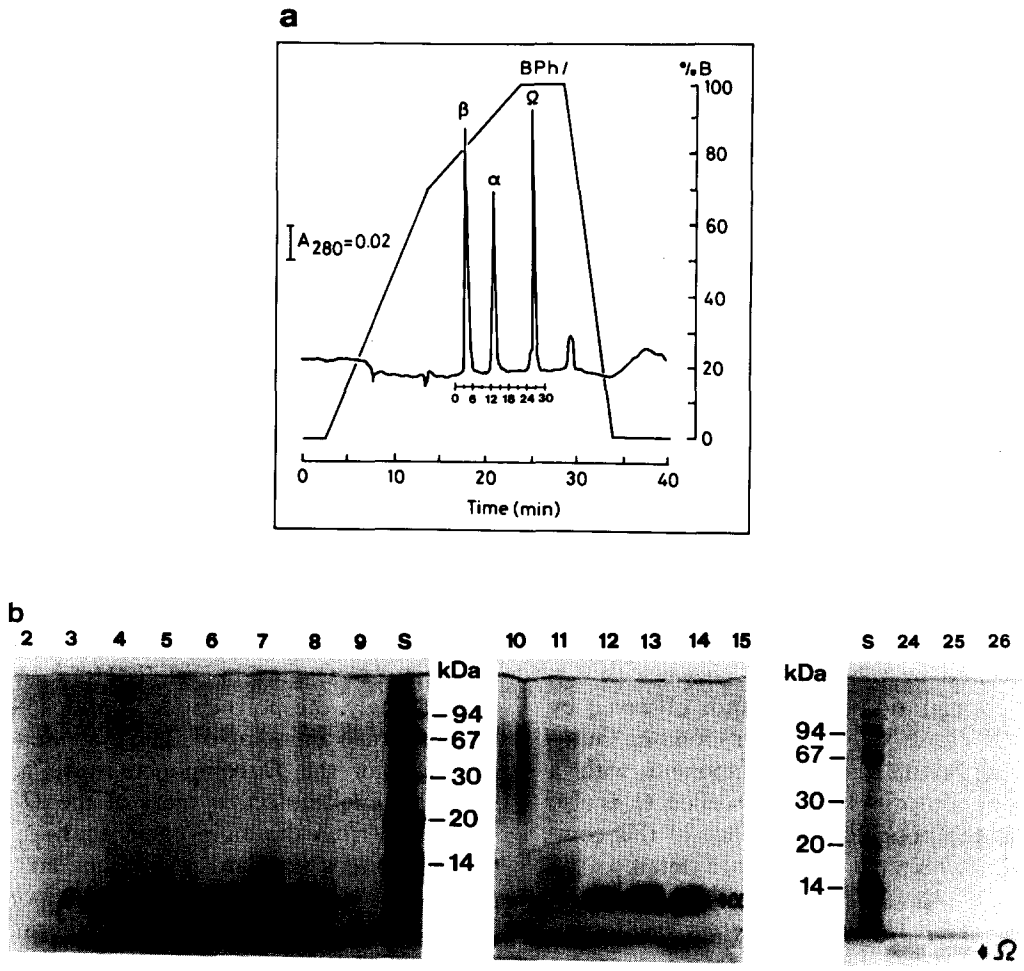

Fig. 3. (a) FPLC chromatography of isolated and solubilized B875 complexes (136 μg protein) from *R. rubrum* G9. The fractions (0.2 ml) collected are shown underneath Peaks I to III and correspond to those shown in (b). (b) SDS-PAGE followed by silver staining of the fractions from Fig. 3(a). The numbers correspond to the fraction numbers shown on the FPLC profile.

B873 kinase were eluted with 5 mM ATP and contained a component which migrated with a molecular mass of approx. 20 kDa when analyzed by SDS-PAGE and silver staining (Fig. 1). Both N_6- and N_8-ATP agarose chromatography were highly efficient, and the elution profiles showed only a few bands on the gel. Purification of the B873 kinase by ATP-Sepharose was not successful; the eluate contained a variety of protein components (data not shown). The use of histone-coupled agarose proved to be impractical; although the B873 kinase could be eluted from the histone affinity column, a large number of other bands were co-eluted (Fig. 1c).

Fractions containing the B873 kinase activity were pooled and then further purified by Sephacryl 300 chromatography. The main peak, containing B873 kinase activity (henceforth called the B873 kinase), contained only a single protein component as judged by SDS-PAGE followed by silver staining, of molecular mass 20 kDa (Fig. 1b). The molecular mass of the native B873 kinase as determined from the Sephacryl 300 step was also 20 kDa, thus suggesting that the B873 kinase is monomeric.

The minute quantities of B873 kinase obtained from the affinity column prevented us from determining the protein concentration after every step. However, 0.8 ml of the pooled B873 kinase after gel filtration used for amino acid analysis (data not shown) contained approx. 990 ng protein. Thus the kinase had been purified 10 000-fold, and the enzyme represents approx. 0.01% of the total soluble protein.

Substrate specificity of the B873 kinase

As observed for the crude supernatant fraction [6], the purified kinase phosphorylated not only the light-harvesting complexes present in the chromatophores but also the highly purified B873 complexes and also histone V-S (Fig. 2). The histones VI, VII, and VIII were also phosphorylated weakly by the B873 kinase (data not shown). No autophosphorylation of the B873 kinase in the absence of added substrates was observed.

Both α- and β-polypeptides of the B873 complexes were phosphorylated by the purified enzyme, as evidenced by the co-migration of the bands observed by SDS-PAGE with those on the corresponding autoradiogram. In addition, a third component with a molecular mass of approx. 4 kDa, here called Ω, is also labelled. Due to the possible importance of this component, we have characterized it in more detail.

Characterization of the Ω component from B873 complexes

When purified B873 complexes were fractionated by FPLC chromatography, three distinct peaks were observed. They were initially assigned according to Ref. 14 as the β-peptide (Peak I), the α-peptide (Peak II) and free bacteriopheophytin (BPh) (Peak III). The latter originates from the acidification of BChl. The identity of Peaks I and II were confirmed by amino acid sequencing. Besides BPh, Peak III contains the Ω component (Fig. 3(a,b)). The Ω component appeared to be extremely hydrophobic, since it eluted at 100% of the eluting solvent and was stained only with the silver reagent but not with Coomassie blue. This latter property may explain why this component has not been observed up to now.

The proteinaceous nature of the Ω component was confirmed by the following physicochemical properties:

(a) It was precipitated by 7% (w/v) TCA; (b) it was precipitated by 75% methanol but was partially soluble in acetone; (c) it did not migrate in a silica gel TLC system (CH_2Cl_2/MeOH/H_2O, 80 : 30 : 4; (v/v/v)) suitable for the separation of phospholipids, aminolipids, and lipopolysaccharides, but remained at the origin. The component at the origin was stained with ninhydrin; (d) it was soluble in 75% (v/v) iPrOH/25% (v/v) H_2O and quantitatively co-eluted with the α- and β-polypeptides when chromatographed on a C4-Sep-Pak cartridge equilibrated in the same solvent. In this solvent the BChl was retained on the column; (e) the FPLC-purified Ω component showed a weak absorption maximum at 28O nm, and was phosphorylated by the purified B873 protein kinase; (f) The FPLC-purified Ω component (Peak III) has been hydrolyzed for the determination of the amino acid composition. However, whereas standard conditions (110°C in 6 M HCl/0.001% phenol for 24 h) were sufficient for the total hydrolysis of the α- and β-peptides, for the Ω component the temperature had to be raised to 160°C to achieve complete hydrolysis, although serine and threonine are degraded almost completely under these conditions (Table II). The reliability of the data was confirmed by simultaneously hydrolysing the α- and β-peptides under the same conditions (data not shown). For the latter, the amino acid compositions obtained were essentially identical to the data obtained at 110°C, except for serine and threonine, which were missing. This unusual resistance of the Ω component to hydrolysis at 110°C may be related to its hydrophobicity. Surprisingly, the polypeptide contains only about 60% hydrophobic amino acids. The cysteine and tryptophan content for the Ω component were not determined. The minimal molecular mass of the Ω-peptide as determined from the amino acid analysis using the data for lysine as a standard, was estimated to be approx. 4 kDa. This value agrees well with the molecular mass as determined by SDS-PAGE. Furthermore, from the amino acid composition data a molar ratio of a : b : Ω of approx. 10 : 10 : 1 was calculated.

It was not possible to obtain any sequence data from the FPLC-purified Ω component even when the de-

TABLE I

Amino acid analysis of the Ω-peptide from R. rubrum G9

The raw data (lanes 1–3) show the results obtained from three pooled (2×100 μl aliquots, see Fig. 3) Ω-containing samples obtained from three independent FPLC profiles. Columns 4–6 and 7 show the molar ratios obtained assuming 1 mol arginine/mol Ω, and the corresponding integer values, respectively.

Amino acid	pmol amino acid determined			pmol amino acid corrected			Integer value
	(1)	(2)	(3)	(4)	(5)	(6)	(7)
Asx	60.5	14.6	20.1	4.4	2.2	2.1	3
Glx	75.5	28.5	33.6	5.4	4.3	3.5	4
Ser	–	–	1.7	–	–	0.2	0
His	4.1	3.5	3.5	0.3	0.5	0.4	1
Gly	84.6	40.6	69.6	6.1	6.1	7.2	6
Thr	3.5	–	1.8	0.3	–	0.2	0
Ala	50.3	18.9	16.4	3.6	2.8	1.7	3
Arg	13.9	6,7	9.6	1.0	1.0	1.0	1
Tyr	1.4	5.0	8.3	0.1	0.7	0.9	1
Val	46.9	10.7	15.0	3.5	1.6	1.6	2
Met	3.4	1.9	3.5	0.2	0.3	0.4	0
Ile	32.0	5.8	7.6	2.3	0.9	0.8	1
Phe	19.2	8.0	10.7	1.4	1.2	1.1	1
Leu	45.6	22.9	25.9	3.3	3.4	2.7	3
Lys	13.8	2.6	11.0	1.0	0.9	1.1	1
Pro	27.4	7.4	8.4	2.0	1.1	0.9	1
Total	82.1	177.1	246.6	34.8	27.0	25.8	28

blocking conditions established for the α-peptide [15] were used.

Metal cofactor specificity

The efficiency with which various metal ions could substitute for Mg^{2+} using purified B873 complexes are as a substrate is demonstrated in Fig. 4. The metal cofactor specificity is essentially identical to that demonstrated for the crude B873 kinase activity [6]. In particular, the activity of the purified B873 kinase in

TABLE II

Determination of the degree of phosphorylation for the isolated polypeptides of the B873 complex

10 nmol (130 μg) B873 complexes were phosphorylated with 120 ng purified B873 kinase in the standard assay. The phosphorylated B873 complexes were dissolved in 1% (w/v) βOG, and chromatographed by FPLC using a PepRPC column. The eluted polypeptides (α, β, ans Ω) were dried and then used directly for the malachite green phosphate determinaton.

Peptide	mol inorganic phosphate/mol polypeptide	
	−kinase	+kinase
α	0.2 ±0.012	0.37±0.01
β	0.14±0.01	0.18±0.02
Ω *	2.3 +1.3	not determined

* The amount of Ω has been estimated assuming the ratio 1 mol Ω/10 mol αβ polypeptides as determined by amino acid analysis.

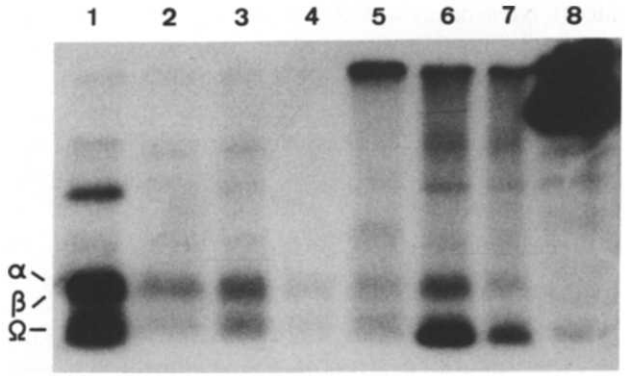

Fig. 4. Metal cofactor specificity of the B873 kinase. Purified B873 complexes (20 μg) were phosphorylated by the homogeneous B873 kinase in the presence of metal cofactors: (1) 10 mM $CuCl_2$; (2) 10 mM $CaCl_2$; (3) 10 mM $MnCl_2$; (4) 10 mM $MgCl_2$; (5) 5 mM $MgCl_2$ + 5 mM $MnCl_2$ (6) 10 mM $ZnCl_2$; (7) 10 mM $MgCl_2$ + 10 mM $ZnCl_2$; (8) the phosphorylation reaction was performed as in (7) and the 5U alkaline phosphatase (Boehringer) was added and the reaction was allowed to proceed for a further 10 min, then analyzed as above. The autoradiogram of the above reactions after analysis by SDS- PAGE is shown.

the presence of transition metals such as Cu^{2+} (Fig. 4, lane 1), Mn^{2+} (Fig. 4, lane 3), and Zn^{2+} (Fig. 4, lane 6), is much larger than that observed for Mg^{2+} (Fig. 5,

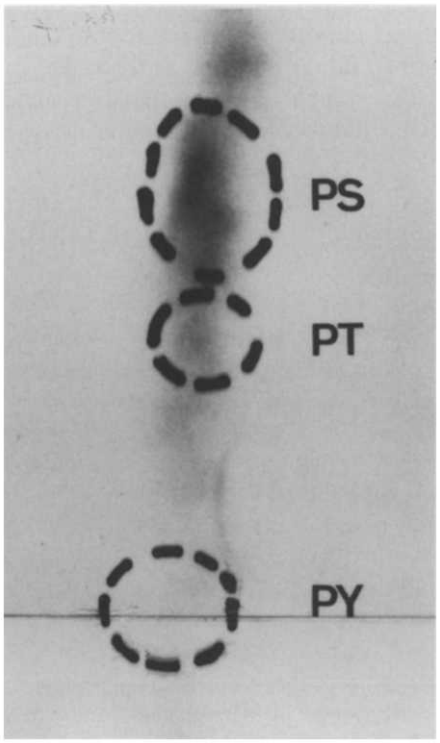

Fig. 5. High voltage electrophoresis and autoradiography of the amino acids phosphorylated by the B873 kinase with histone V-S (32 nmol) as the substrate. Phosphoserine (PS), phosphothreonine (PT), and phosphotyrosine (PY) standards were separated in the same hydrolysis mixture and stained with ninhydrin (dotted lines). The corresponding autoroadiogram is shown.

lane 5). No synergy was observed when metal ions were combined in sub-optimal concentrations (e.g., Mg^{2+} and Mn^{2+}, Fig. 4). Ca^{2+} only weakly activated the B873 kinase. The further addition of alkaline phosphatase subsequent to the phosphorylation reaction in the presence of added Zn^{2+} led to extensive dephosphorylation of the α, β, and Ω components of the isolated B873 complex (Fig. 4, lane 8).

Determination of the phosphorylated amino acids

The amino acid(s) phosphorylated by the B873 kinase were identified after partial acid hydrolysis of a phosphorylated substrate followed by high voltage electrophoresis. However, the hydrolysis products from the B873 complex always led to a poorly defined smear on the autoradiogram, probably due to the well-known resistance of very hydrophobic polypeptides to short acid hydrolysis. For this reason, the determination of the phosphorylated amino acid rests solely on the data obtained when histone V-S was employed as a substrate. In this case (Fig. 5), we detected repeatedly a labelled spot on the autoradiogram which comigrated with the phosphoserine standard.

In order to eliminate tyrosine as the phosphorylation site, we analyzed various cell fractions by immunoblotting, using a commercially available monoclonal antibody against phosphotyrosine. As shown in Fig. 6 (lanes 2–5) although various phosphotyrosine proteins were observed in both chromatophore and supernatant fractions of *R. rubrum* as well as from *Rb. sphaeroides* (included for comparison), no positive signal in the region of the light-harvesting complexes of either organism was detected. By comparison, a strong positive signal was observed for the control, phosphotyrosine-coupled ovalbumin. This negative result was confirmed by a dot blot, where larger amounts of protein, including a sample of purified, phosphorylated B873 complexes, were employed (Fig. 6b).

To determine the stoichiometry of B873 phosphorylation, purified B873 complexes, phosphorylated in vitro with the isolated kinase, were chromatographed using FPLC and the amount of bound phosphate determined using the malachite green method (Table II). The phosphate determination was also performed for B873 complexes which had not been phosphorylated in vitro (Table II). Both α- and β-polypeptides were significantly phosphorylated (one-fifth and one-seventh of the total, respectively (Table II)) in the purified complexes, and this can be increased by in vitro phosphorylation using the purified B873 kinase. Table II indicates that at least 20% of the isolated B873 complexes are phosphorylated and up to one-third of the complexes are phosphorylated in vitro.

Discussion

Protein phosphorylation of the B873 light-harvesting complexes from *R. rubrum* has been demonstrated by several groups and has been implicated in the regulation of energy transfer between individual photounits [16,17]. However, the detailed characterization of the protein kinase(s) involved has not been performed until now.

For the first time, a protein kinase capable of phos-

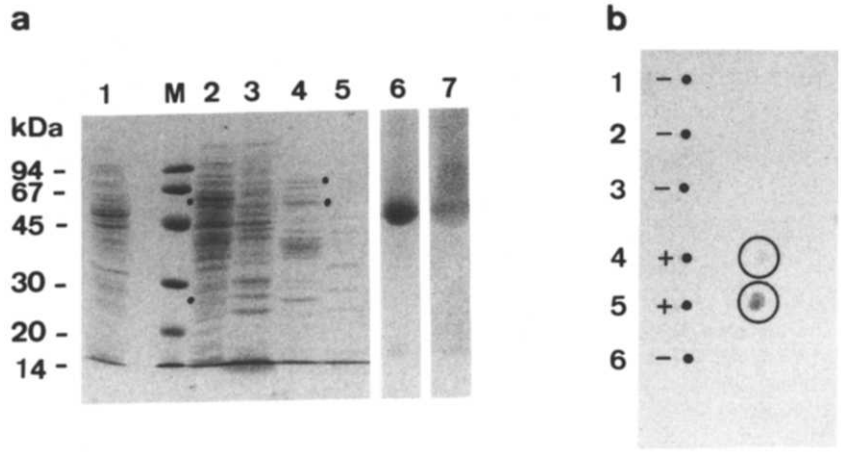

Fig. 6. Immunoblotting of phosphorylated cell fractions (35 μg each) using an anti-phosphotyrosine monoclonal antibody. (a) Western blotting of: (1) *E. coli* crude extract; (2) water-soluble fraction from *Rb. sphaeroides*; (3) chromatophore fraction from *Rb. sphaeroides*; (4) water-soluble fraction from *R. rubrum*; (5) chromatophore fraction from *R. rubrum*; (6) phosphotyrosine-linked ovalbumin (20 mg). For fractions 1–6 the Coomassie blue-stained gel is shown and the bands which gave a positive signal after Western blotting are indicated. Lane (7) shows the Western blot of lane 6 as a positive control. (b) Dot blot of various phosphorylated samples using an anti-phosphotyrosine monoclonal antibody: (1) *E. coli* crude extract (30 μg); (2) phosphorylated B873 complexes (30 μg); (3) ovalbumin after phosphorylation in the standard assay (30 μg); (4) phosphotyrosine-linked ovalbumin (15 μg); (5) phosphotyrosine-linked ovalbumin (30 μg); (6) phosphotyrosine (100 nmol). Positive (+) and negative (−) signals are indicated. The negative signal observed for phosphotyrosine alone is probably due to its weak binding to nitrocellulose filters and subsequent removal in the wash procedure.

phorylating homogeneous B873 complexes in vitro has been purified from *R. rubrum*. This B873 kinase is present in very low amounts in the cell (approx. 0.01% of the total cellular protein), is monomeric with a molecular mass of 20 kDa, and water-soluble. The B873 kinase appears to be one of the smallest functional protein kinases isolated from procaryotes so far [18]. In vitro phosphorylation of purified B873 complexes with the isolated kinase showed that all subunits, the α-, β-, and the new component, Ω, are phosphorylated, probably at one or more serine residues. This observation, together with the fact that the α-subunit has been shown to be phosphorylated after isolation from whole cells [6,16], would imply that the B875 kinase is localized in the periplasmic space, where the only serine residues of the α-polypeptide are present. For several *Escherichia coli* proteins, phosphorylation appears to occur at the conserved sequence -D/E-X-X-S- [1]. However, this sequence is not observed for the α- or the β-polypeptide. Furthermore, the putative consensus sequences deduced by Allen [3] for the L and M polypeptides of the reaction center, and D1 and D2 proteins of photosystem II are also absent in the light-harvesting α- and β-polypeptides. The conclusion that the phosphorylation of the B873 complexes occurs on the periplasmic side still holds if either threonine or tyrosine are phosphorylated. Interestingly, both α- and β-polypeptides contain a serine residue at the last position of the periplasmically located C-terminus. In fact the suggestion that phosphorylation events occur in the periplasm is not novel; in *E. coli* there exists a periplasmic protein, the arginine-ornithine binding protein which has been suggested to be regulated by phosphorylation [19], although this observation has not gained general acceptance. Attempts in our laboratory to obtain unequivocal evidence for the localization of the kinase by the isolation of a periplasmic fraction not contaminated with traces of cytosolic components have been unsuccessful so far. Thus, we cannot yet distinguish unequivocally between the assignment of kinase to the periplasm and an artefactual phosphorylation in the in vitro system.

Surprisingly, no evidence was found for a phosphotyrosine in either chromatophores or in in vitro phosphorylated B873 complexes. This is in contrast to the studies of Vallehos et al. [20,21], who found phosphotyrosine in the membrane fractions of *R. rubrum*, and have partially purified a tyrosine kinase activity. The reason for this difference is not clear at present. Furthermore, phosphorylation was attributed to a 13-kDa band isolated from B873 complexes. To our knowledge, the 13 kDa band corresponds to the α-polypeptide, which contains no tyrosine. It may also be that the tyrosine kinase activity is highly unstable and was simply not observed in the present study. In any case, the tyrosine kinase of the membrane fraction was unable to phosphorylate histones and must be disinct from the B873 kinase observed here. The B873 kinase activity is also independent of the presence of cAMP and phosphorylates histones, thus resembling the protein kinase C family of eucaryotes [5,18]. So far we have not been able to demonstrate autophosphorylation, nor does the enzyme catalyse a dephosphorylation of phosphorylated B873 complexes. However, the kinase-phosphorylated B873 complexes are easily dephosphorylated by the addition of alkaline phosphatase, suggesting that the phosphoryl moiety remains accessible to the medium.

Although a number of different kinases may be involved in phosphorylation of the B873 complexes in vivo, the protein kinase activity characterized here seems to be primarily responsible for the regulation of energy transfer between B873 units for the following reasons: (a) the B873 kinase can be purified by affinity chromatography under conditions corresponding to those yielding maximal phosphorylation of the intact chromatophore membrane; (b) all three components (α-, β- and Ω-polypeptides) of purified B873 complexes become phosphorylated by the purified kinase; (c) the activity of the B873 kinase in the presence of different metal ions is identical to that observed for the crude supernatant fraction [6]. In particular, transition metal ions are more effective than Mg^{2+} for activating the kinase, a property which is reminiscent the metal ion specificity of pyrophosphatases found in *R. rubrum* [22]; the addition of either ADP or AMP shows only a weak inhibition of the kinase, similar to the supernatant fraction obtained from crude extracts [6].

Finally, the present study with purified B873 complexes and B873 kinase has revealed a previously undetected component, Ω, which appears to be associated with the B873 complex and may be involved in regulating energy transfer between complexes. So far, the light-harvesting complexes from purple non-sulphur bacteria have been assumed to be composed of two non-identical peptides, α and β, which bind BChl and carotenoids [1,23–26]. However, there are suggestions that there might be a third component in the light-harvesting complexes:

(a) Despite the extensive homology between the α- and β-peptides of *R. rubrum* and *Rhodopseudomonas viridis*, respectively, the BChl *b*-containing light-harvesting complex (B1050) from the latter organism contains a third peptide, γ, which has been suggested to be responsible for the large red-shift to 1050 nm [27]. This γ-peptide is strikingly similar to Ω; it is not stained by Coomassie blue, it is composed of 36 amino acids yielding a molecular mass of approx. 4 kDa and is also extremely hydrophobic. In contrast to Ω however, the γ-peptide is present in an equimolar ratio to the α- and β-peptides, respectively [27,28].

(b) Van Grondelle and co-workers [29] have detected spectroscopically a pigment species absorbing at 896 nm (B896) which might be bound to a hitherto unknown protein and which they suggest to be mediating energy transfer between the B875 (B880) and the reaction centre (RC) in *R. rubrum*, *Rb. sphaeroides*, and *Rb. capsulatus*, respectively. In all cases the ratio of B896/RC ratio was approx. 1–2, thus setting the B880/B896 ratio to approx. 6–12, a stoichiometry similar to the one of $(\alpha + \beta)/\Omega$ observed here.

(c) Two unknown open reading frames, pufQ and pufX, found in the puf operon of *Rb. sphaeroides* and *Rb. capsulatus*, would lead to proteins of apparent molecular masses of approx. 8 kDa and 9 kDa, respectively [30,31]. PufQ has been implicated in the regulation of BChl and haem synthesis, whereas pufX is thought to play a role in the assembly of reaction centres into the chromatophore membrane [30–33]. In both cases the gene products are not known and their functions remain controversial.

(d) In contrast to the γ-peptide from *Rps. viridis*, the Ω-peptide isolated from B875 complexes from *R. rubrum* is phosphorylated by a water-soluble protein kinase which is accompanied by a decrease in the intrinsic fluorescence of the B875 complexes in chromatophores [6]. These observations suggest a role of the Ω-peptide in the regulation of light-energy transfer between B875 complexes.

We believe that the Ω polypeptide is tightly linked to the B873 complexes: it is always present in highly purified preparations of B873 complexes from various strains of *R. rubrum* in a fixed ratio to α- and β-polypeptides and we have also detected a component with similar characteristics in chromatophore preparations of *Rb. sphaeroides* 2.4.1 (unpublished data). An unequivocal functional assignment of Ω, however, must await the assignment of its genetic locus and subsequent deletion.

Acknowledgements

We acknowledge the support by the Swiss National Science Foundation (Grant 31.25628.88). We also thank Dr. P. Hunziker (Institute of Biochemistry, University of Zurich,) and Dr. P. Jenoe, (Department of Biochemistry, Biocenter, Basel) for performing the amino acid analyses, and Dr. H. Lahm (Hoffmann-La Roche Ltd., Basel) for performing the amino acid microsequencing.

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