Membrane-bound Phosphate as Driving Force for ATP Synthesis in Chromatophores of *Rhodospirillum rubrum*

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Abstract

Chromatophores of *Rhodospirillum rubrum* preilluminated in the presence of unlabelled phosphate form labelled ATP in the dark after being separated from the preincubation mixture by gel-filtration and incubated with ADP and $^{32}P_i$. The driving force for the synthesis of this labelled ATP was previously shown to be ATP firmly bound to the membrane. The amount of labelled ATP produced is determined by measuring the incorporated $^{32}P_i$ and extrapolation of the values towards zero time incubation and is shown to correlate with the phosphorylation activity of the chromatophores used. 2.5 nmoles ATP correspond to a phosphorylation activity of 100 μmoles ATP/mg Bchl . h. The results were compared with those from assays in which chromatophores were preincubated with pyrophosphate in the dark. The results strongly support the hypothesis that the $X \sim P$ formed in the light does not transfer its P to ADP in solution and therefore give further evidence to a double sited coupling factor as proposed previously.

Introduction

Keister and Minton [1] demonstrated a pyrophosphate driven ATP synthesis in chromatophores of *Rhodospirillum rubrum*. In this reaction the γ-P of ATP originates from free phosphate in the solution [2] rather than from pyrophosphate. It was shown recently in our laboratory [3]

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that the mechanism of photophosphorylation in *R. rubrum* might be strikingly similar. By using centrifugation techniques to separate chromatophores from the preincubation mixture a membrane bound phosphate [4, 5], later shown to be firmly bound ATP [3], was found in the centrifuged chromatophores. Nucleotides bound to the coupling factor were previously demonstrated in chloroplasts by Roy and Moudrianakis [6]. In addition Yamamoto *et al.* [7] were able to show that phosphorylating chromatophores of *R. rubrum* first produce ATP bound to the chromatophores from endogeneous ADP. Previously we demonstrated that firmly bound ATP produces free ATP in a subsequent dark incubation, thus proving its X ~ P nature. The firmly bound ATP decreases during dark incubation with added ADP and gives rise to acid soluble ATP. The γ-P from firmly bound ATP, however, is not transferred to the added ADP [3]. The time elapsed between the light or dark preincubation and the dark incubation in the presence of ADP was up to 60 min. The established half life of the X ~ P in chloroplasts is a few seconds [8] for a two stage phosphorylation and 10-30 min for the ATP-P_i exchange induction [9]. It was not surprising therefore to find no light stimulation in normal chromatophores. Any X ~ P formed in the light might have reacted with loosely bound nucleotides while being deenergized [7, 10]. A rapid separation of the P-loaded chromatophores from the preincubation mixture should, however, increase the life time of X ~ P by depletion of suitable substrates. Such a result was only achieved with centrifugation using EDTA treated, partially uncoupled and later reconstituted chromatophores.

In this investigation the time between preincubation and dark incubation of the chromatophores with ADP is shortened by separating the membranes from the reaction mixture using a Sephadex column G-25. With this method we are able to confirm the observations that chromatophores preilluminated in the presence of unlabelled phosphate, separated from the preincubation mixture and incubated in the dark with $^{32}P_i$ and ADP, produce labelled ATP.

Methods

Chromatophores from *R. rubrum* S-1 were prepared as described previously [3] except that before washing they were treated with nucleases to remove adsorbed or bound nucleic acids. Chromatophores were incubated at room temperature under nitrogen for 30 min in 0.1 M HEPES-NaOH (pH 7.0), 0.2 M sucrose, 10 μg DNase and 10 μg RNase per mg bacteriochlorophyll. Bacteriochlorophyll was determined using the *in vivo* extinction coefficient reported by Clayton [11]. Phosphorylation activity of the chromatophores was measured under aerobic conditions in open tubes at 30°C in a total volume of 1 ml, containing

100 μg bacteriochlorophyll/ml, 5 mM ADP (pH 8.0), 5 mM $MgCl_2$, 0.5 mM Na-succinate, 5 mM $^{32}P_i$ (pH 8.0), 1 mg/ml serum albumin, 0.1 M HEPES-NaOH (pH 8.0). The samples were incubated for 5 min and the reactions were stopped with perchloric acid, final concentration 2%. Net phosphorylation activity is the difference between light and dark incubations. The total $^{32}P_i$ esterified was measured according to Avron [12]. Inorganic phosphate in the chromatophores separated from the preincubation mixture was determined as described by Datta and Franklin [13]. Radioactivity was measured in a Nuclear Chicago Model 724 Scintillation counter in aqueous solutions using Cerenkov emission [14]. The values were corrected for quenching according to the channel ratios. Preincubated chromatophores were separated from the preincubation mixture by chromatography over a Sephadex G-25 coarse column 2.7 x 20 cm, with a sample volume of 3.0 ml. The column was equilibrated and eluted with Tris-HCl (0.1 M, pH 8.0) at a flow rate of 3.5 ml/min. Incubations of the separated chromatophores were performed immediately in the dark as described in the results. Perchloric acid was added to a final concentration of 5% to stop the reaction. The samples were chilled in liquid nitrogen and stored below $-20°C$ until analysis. Chromatographic separations were performed as previously described on DEAE Sephadex A-25 [3]. We assume that the labelled phosphate added during incubation equilibrated with the inorganic, unlabelled phosphate still adsorbed after separation from the preincubation mixture. To calculate the specific activity of the $^{32}P_i$ during incubation, the adsorbed unlabelled P_i was determined in each preparation. The amount of adsorbed phosphate varied within the range of 200-300 nmoles/mg bacteriochlorophyll for preilluminated chromatophores and of 400-500 nmoles/mg bacteriochlorophyll for dark preincubated ones. The amount of labelled esterified phosphate was calculated from the cpm incorporated and the specific activity of the inorganic phosphate.

Chemicals

All chemicals used were of the highest purity. $^{32}P_i$ was purchased from the Eidg. Inst. für Reaktorforschung Würenlingen and contained up to 2% labelled PP_i. $^{32}PP_i$ came from the Radiochemical Center Amersham. Pancreatic RNase and DNase (B grade) was bought from Calbiochem Los Angeles.

Results

Chromatophores were preincubated in the light with unlabelled phosphate in the absence of added ADP. After separation from the

preincubation mixture the particles were incubated with labelled inorganic phosphate and ADP at pH 8.0. The esterification capacity of the preincubated chromatophores is defined as the amount of esterified phosphate in the supernatant after stopping the reaction with perchloric acid and is shown in Fig. 1 as a function of incubation time. Following a lag phase, labelled esterified phosphate increases to a constant level reached after about 2 hours. The formation of ATP from added ADP by adenylate kinase [15] combined with an ATP-P_i exchange reaction gives rise to the large amount of labelled ATP detected after long incubations. On the other hand from zero min up to five min there is a constant low amount of $^{32}P_i$ esterified which does not seem to be the result of these
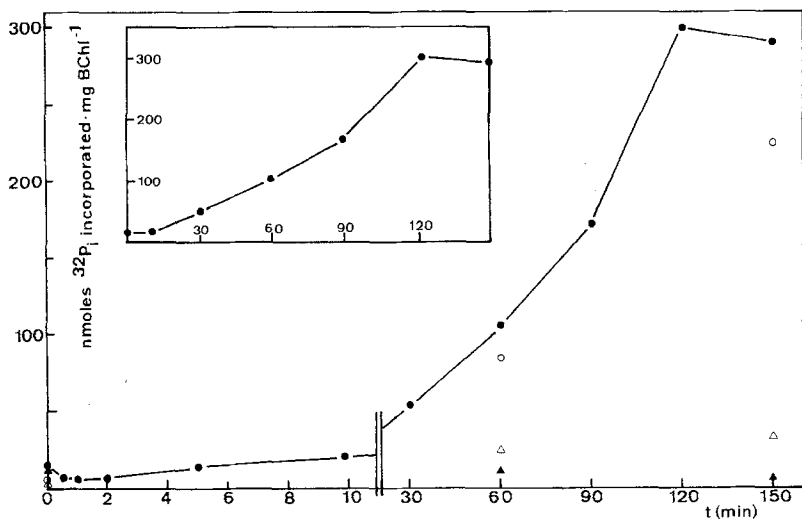

Figure 1. Time course of $^{32}P_i$ incorporation into perchloric acid soluble supernatant of chromatophores preincubated in the light with unlabelled phosphate and incubation with $^{32}P_i$ and ADP after separation of the chromatophores from the preincubation mixture. Control experiment not subtracted. Preincubation in the light for 5 min 30°C in a total volume of 5 ml containing 5 mM KCl, 2 mM sodium succinate, 7.5 mM phosphate (pH 8.0), 0.1 M Tris-HCl buffer (pH 8.0), chromatophores 500 μg BChl/ml. Incubation after separation from the preincubation mixture as given in Methods in a total volume of 2 ml containing 2 mM $MgCl_2$, 0.1 mM sodium succinate, 1 mg/ml serum albumin, 2 mM ADP (pH 8.0), 0.1 M HEPES-NaOH (pH 8.0), 0.2 mM $^{32}P_i$ (pH 8.0) 1.5 x 10^7 cpm/ml, chromatophores 50 μg BChl/ml. After the column separation the chromatophores were added to the incubation mixture containing either labelled phosphate and perchloric acid for zero time incubations or only labelled phosphate and the incubation mixture for all other incubation times. Phosphorylation activity of the chromatophores used: 24 nmoles P_i esterified/mg BChl . h.
●——●, total $^{32}P_i$ esterified; ○, $^{32}P_i$ incorporated into ATP; △, $^{32}P_i$ incorporated into ADP; ▲, $^{32}P_i$ incorporated into PP_i.

two reactions. To exclude non-specific formation of ATP, controls were run in which serum albumin was substituted for chromatophores. Phosphorylation values of these controls were subtracted from the value of the experiments with chromatophores. The amounts of $^{32}P_i$ incorporated in these controls remained constant up to 10 min incubation, but individual samples varied and gave up to 0.2 nmoles P_i esterified per ml compared to 0.3-0.4 nmoles P_i esterified per ml in the presence of chromatophores. On the other hand incubations of albumin with phosphorylation mixtures at room temperature for 2 days led to considerable incorporation of P_i into nucleotides in the range of 10-100 nmoles P_i per ml.

The differences measured are relatively small. When the necessary controls were subtracted, the extrapolation back to zero time of the amount of ^{32}P-ATP (= partial esterification capacity) should indicate the amount of acid soluble ATP able to be synthesized with the aid of the firmly bound ATP. In experiments (Fig. 1-3) the samples stopped at zero time incubation showed as high or higher values of total P_i esterified than after 15 or 30 secs. As can be seen from Fig. 2 and 3 the initial decrease of the esterification capacity within the first 15 secs is due to a
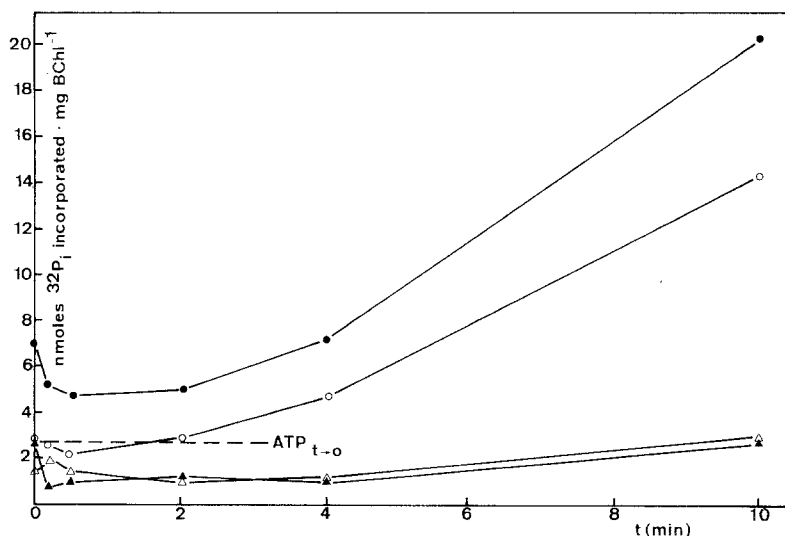

Figure 2. Partial esterification capacity after light preincubation in the presence of unlabelled phosphate.

Preincubation medium as given in Figure 1, incubation medium as given in Figure 1 too, except labelled phosphate contained 9.1×10^6 cpm/ml. The phosphorylation activity of the chromatophores used was 87 μmoles P_i esterified/mg BChl.h.
●——● total $^{32}P_i$ esterified; ○----○ $^{32}P_i$ incorporated into ATP; △——△ $^{32}P_i$ incorporated into ADP; ▲——▲ $^{32}P_i$ incorporated into PP_i.
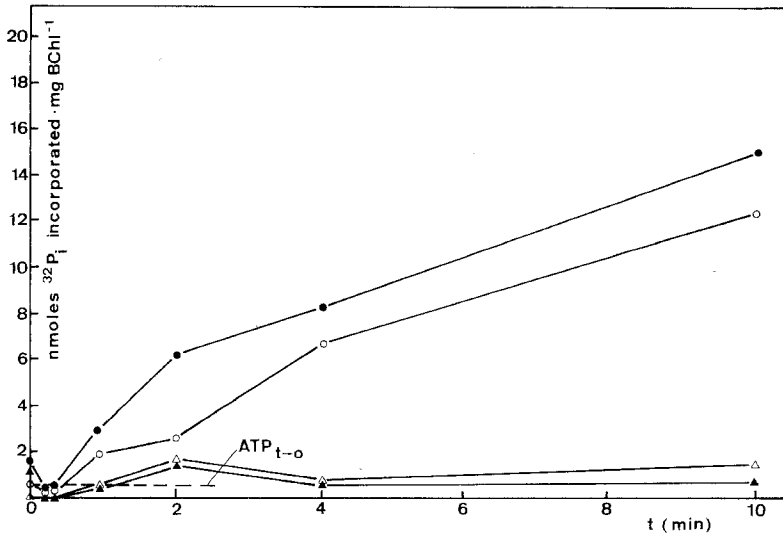
Figure 3. Partial esterification capacity after dark preincubation in the presence of unlabelled phosphate.

Conditions as given in Figure 2. The experiment was done at the same day and with the same chromatophore preparation as that shown in Figure 2.

●—● total $^{32}P_i$ esterified; ○—○ $^{32}P_i$ incorporated into ATP; △—△ $^{32}P_i$ incorporated into ADP; ▲--▲ $^{32}P_i$ incorporated into PP_i.

decrease of the labelled pyrophosphate in both the dark and light preincubated samples. Figure 2 shows the partial esterification capacity when chromatophores were preincubated in the light in the presence of unlabelled phosphate before being separated on Sephadex and incubated with $^{32}P_i$ and ADP. Light preincubation produced ten times more labelled ATP than dark preincubation. The amount of pyrophosphate at zero time incubation is actually much smaller than shown, because it represents labelled pyrophosphate originally present in the labelled phosphate solution and its amount was calculated using the specific activity of the inorganic phosphate which was diluted by the adsorbed unlabelled phosphate. Despite the overestimation of its amount the pyrophosphate hydrolized during the first seconds of incubation is hardly sufficient to form the ATP detected, assuming the stochiometry of 1 ATP per 12 PP_i as found by Keister and Minton [1, 2].

In a set of different experiments the partial esterification capacity of light preincubated chromatophores was compared to the phosphorylation activity of the chromatophores used. There is a good correlation between the 2 values (Table I). In the experiment shown in Fig. 4 the chromatophores were preincubated with pyrophosphate in the dark. Chromatophores energized with PP_i have twice the partial

TABLE I. Correlation between partial esterification capacity and the
phosphorylation activity of the chromatophores used

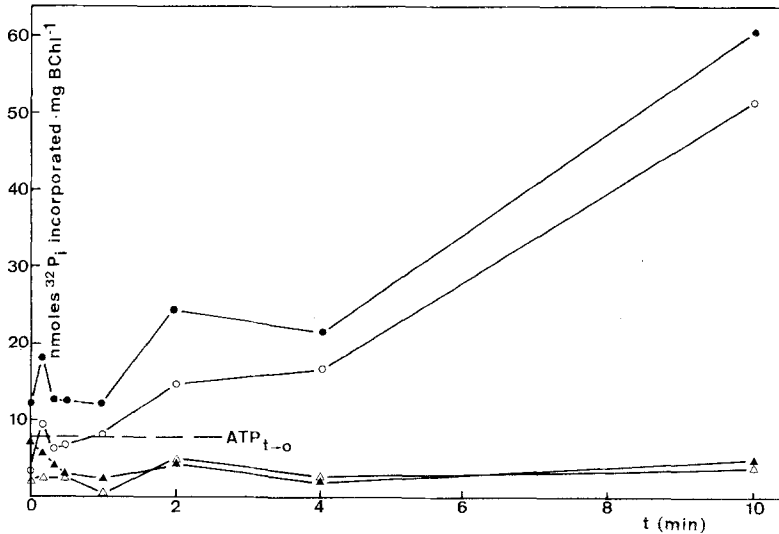|	Phosphorylation activity μmoles P_i esterified per mg BChl and h	Partial esterification capacity nmoles P_i esterified per mg BChl
Experiment 1	189	4.0
Experiment 2	81	2.0
Experiment 3	65	1.8

Conditions as given in figure 1.

Figure 4. Partial esterification capacity after preincubation with pyrophosphate in the dark.

Conditions as given in Figure 2, except that 5 mM PP_i was added instead of phosphate during preincubation in the dark. The experiment was done at the same day and with the same chromatophore preparation as that shown in Figure 2.
●――● total $^{32}P_i$ esterified; ○――○ $^{32}P_i$ incorporated into ATP; △――△ $^{32}P_i$ incorporated into ADP; ▲――▲ $^{32}P_i$ incorporated into PP_i.

esterification capacity of those preincubated with P_i in the light. The amount of labelled ATP increases markedly during the following seconds to an almost constant level after 1 min. This may be due to a further energization of the chromatophores by unlabelled pyrophosphate molecules still adsorbed to the membranes after the separation from the preincubation mixture. Experiments in which chromatophores were preincubated with labelled phosphate and subsequently incubated with

ADP and unlabelled P_i were run parallel to some of the above described experiments. The data are difficult to interprete because it is impossible to distinguish between the decrease in activity expected as an effect of isotopic dilution and the decrease in the labelled esterified phosphate due to simple hydrolysis. Nevertheless results of the normal type of experiments $P_i \rightarrow {}^{32}P_i$, (Table II) show clearly that practically no hydrolysis of the total ${}^{32}P_i$ esterified takes place during the first five minutes. Since the constant level of the total esterified phosphate in the $P_i \rightarrow {}^{32}P_i$ experiments does not result from the action of adenylate kinase and ATP-P_i exchange reaction, the decrease of the total ${}^{32}P_i$ incorporated during the first five minutes of incubation in an ${}^{32}P_i \rightleftharpoons P_i$ experiments must be due to an isotopic dilution.

TABLE II. Total ${}^{32}P_i$ esterified before and after incubation in experiments using either unlabelled or labelled P_i during preincubations and incubations

Conditions		Total ${}^{32}P_i$ esterified in nmoles P_i per mg BChl	
Preincubation	Incubation	$t = 0'$	$t = 5'$
${}^{32}P_i$ light	P_i dark	3.9	1.0
P_i light	${}^{32}P_i$ dark	0.5	1.4
${}^{32}P_i$ dark	P_i dark	1.1	0.7
P_i dark	${}^{32}P_i$ dark	0.6	0.6

Conditions for preincubation as given in Fig. 1. The incubation mixture contained in a total volume of 2 ml 2 mM $MgCl_2$, 0.1 mM sodium succinate, 1 mg/ml serum albumin, 2 mM ADP (pH 8.0), 0.1 M Hepes-NaOH (pH 8.0), ${}^{32}P_i$ 10^{-5} M or P_i 10^{-5} M respectively, chromatophores 30 μg BChl/ml. The phosphorylation activity of the chromatophores was 153 μmoles P_i esterified per mg BChl and h.

Discussion

The results described are fully consistent with our previously proposed mechanism [3]. The data support the assumption that preilluminated chromatophores in the presence of phosphate form a membrane bound $X \sim P$. This product is able to produce acid soluble, labelled ATP when incubated with labelled phosphate and ADP after the separation of the membranes from the preincubation mixture. Due to the short separation procedure a light stimulated esterification capacity could be demonstrated with normal chromatophores which was not possible in the experiments using zonal centrifugation. On the other hand the amount of adenylate kinase partially bound to chromatophores [15] is higher after column chromatography than after centrifugation in a density

gradient. Furthermore with the former method the amount of this enzyme remaining on the membrane varied as can be seen from the time difference of the point of increase of the total $^{32}P_i$ esterified. Since the amount of ATP formed after zero time incubation is similar to that formed after 1-2 min incubation, the synthesis of ATP may possibly proceed by a one step reaction which is probably completed with the time necessary for complete denaturation of the chromatophores. Assuming a total amount of coupling sites equivalent to 20-30 nmoles ATP per mg Bchl [16, 17], the half life can be calculated to be about 10 min using the remaining amount of ATP after 30 minutes.

The appearance of label in ADP (Fig. 2-5) during the first seconds still can not be explained by our hypothesis. An early labelling of ADP would be expected according to the mechanism proposed by Roy and Moudrianakis [6] for chloroplasts, but our findings differ substantially from theirs with respect to the origin of the γ-P of the ATP.

In Boyer's [18] and Slater's [19] recent hypothesis of the coupling mechanism in mitochondria, firmly bound ATP is also involved. Bound ATP is formed in a hydrophobic environment without the use of metabolic energy while the latter is necessary to remove the ATP from the membrane. Similarly we proposed [3] that one site of the coupling factor is usually loaded with ATP which is not released unless the coupling factor is energized again either with another ATP or with light in the presence of ADP. In our hypothesis we assume a double sited coupling factor in which the energy is still needed to form a new ester bond. The firmly bound ATP formed during preincubation does not become free but drives the esterification of a second ADP and P_i during incubation in the dark while being hydrolized. Our concept of a two site coupling factor differs therefore from other recently published schemes in that it does not involve a direct P_i transfer from the firmly bound nucleotides, representing the $X \sim P$, to the added ADP.

The experiments presented by Cross et al. [20] and Boyer et al. [18] show in principle, the same results for submitochondrial particles as Yamamoto et al. [7] demonstrated for chromatophores of *R. rubrum*, giving further evidence of the similarities of the two energy transfer systems. Considering the present knowledge of the mechanism of phosphorylation, it is probable that there does not exist a true chemical phosphorylated intermediate able to transfer its P to added ADP. The first detectable phosphate ester seems so far to be bound ATP [3, 7, 18, 19, 20] which may be able to store the conformational energy in the form of an acyl ester bond buried in the coupling factor protein. This bound ATP could represent a phosphorylated product with energy buffer capacity and with a much longer life time than that of the energized conformation itself.

Possible supports to our findings and unknown similarities might come

from work performed on *Streptococcus faecalis*. Abrams *et al.* [21] could show by zonal centrifugation that the plasma membrane bound Mg^{+2} dependent ATPase has tightly bound nucleotides. These authors measured approximately one ATP and one ADP per mol of enzyme.

Acknowledgements

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