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SYNTHESIS OF FREE ATP FROM MEMBRANE-BOUND ATP IN CHROMATOPHORES OF *RHODOSPIRILLUM RUBRUM*

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SUMMARY

Chromatophores of *Rhodospirillum rubrum* were preincubated with $^{32}P_i$ in the absence of added nucleotides. Particles and reaction mixture were then separated by sucrose density gradient centrifugation. The labeled chromatophores thus obtained esterify $^{32}P_i$ into acid-soluble ATP (ATP_{as}) on the addition of ADP in the dark. Additional firmly bound ATP (ATP_{fb}) can be liberated on sodium dodecylsulfate treatment. Coinciding with the formation of acid-soluble ATP there is a decrease in the amount of firmly bound ATP. The isotopic concentration experiments in which labeled chromatophores were incubated with carrier-free $^{32}P_i$ and ADP in the dark, show that ATP_{as} might arise from ATP_{fb} not by a direct γ-phosphate transfer but by an esterification of the added ADP and free phosphate with a concomitant hydrolysis of the ATP_{fb}. On this basis we have proposed a new working hypothesis for the last step of electron transport-linked phosphorylations. It includes the following reactions:

$$\sim + P_i^* \longrightarrow \sim P^* \text{ (i.e., } ATP_{fb})$$
$$\sim P^* + ADP + P_i^{**} \longrightarrow ATP_{as}^{**} + P_i^*$$

The hypothesis is compatible with the concept of conformational energy conservation.

INTRODUCTION

In 1966 Horio et al. [1] suggested that $X \sim ADP$ may be a possible high energy phosphorylated intermediate in *Rhodospirillum rubrum*. Recent results on the coupling factor of chloroplasts show that nucleotides are bound to the coupling factor itself and can be solubilized from precipitated protein by urea (Roy and Moudrianakis, [2]). Our previous work on photophosphorylation in the absence of added nucleotides (Lutz, [3]) demonstrated that ADP and ATP are labeled along with the major

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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product pyrophosphate [4]. Labeled ADP and ATP probably originate from bound nucleotides since the chromatophores were washed before being incubated with $^{32}P_i$. These findings have led to an investigation of the possible membrane-bound phosphorylated intermediates using zonal centrifugation [5]. With this method large amounts of chromatophores can be separated from free $^{32}P_i$ and depleted of loosely adsorbed reaction products such as pyrophosphate. The isopycnically centrifuged chromatophores contain $^{32}P_i$ which, as shown by ultrafiltration, is not only adsorbed but partially bound to the chromatophore membrane [6, 7]. Part of this bound phosphate is acid-soluble esterified phosphate [5, 8]. When labeled chromatophores are incubated in the dark in the presence of ADP the amount of acid-soluble esterified phosphate increases under the appropriate conditions [8].

The present investigations deals with the firmly-bound ATP (ATP_{fb}) as a possible precursor of the acid-soluble esterified phosphate (ATP_{as}). Evidence is presented which shows that ATP_{as} arising from ATP_{fb} on ADP addition does so by esterifying free P_i and ADP with a concomitant hydrolysis of ATP_{fb}. A γ-phosphate transfer from ATP_{fb} to ADP does not seem to occur.

METHODS

Coupled and uncoupled chromatophores from R. rubrum

R. rubrum S1 cells were grown anaerobically in the light for 48 h on a chemically defined malate medium [9]. Normal chromatophores were prepared by grinding the cells with aluminium oxide [10] in 0.05 M Tris–HCl, pH 8.0. The chromatophores were taken as the cell fraction which sedimented between 25 000 $\times g$ for 30 min and 140 000 $\times g$ for 90 min. The preparation of partially uncoupled chromatophores and a coupling factor was based on the methods of Baccarini-Melandri et al. [11] and Johansson [12]. These preparations were assayed for photophosphorylating activity on the same day of their use. Bacteriochlorophyll was determined using the in vivo extinction coefficient of 140 $mM^{-1} \cdot cm^{-1}$ at 880 nm as reported by Clayton [13].

Principle of the centrifugation experiments

Chromatophores were preincubated in the dark, at 30 °C with $^{32}P_i$ under constant agitation and purified nitrogen. The composition of the preincubation mixtures is given for each experiment in the results section. The sample was illuminated with red light (wavelength greater than 580 nm) with three 300 W lamps. The chromatophore suspension was then transferred on the top of the gradient in the rotor. Zonal centrifugation was performed in a Spinco L 50 centrifuge with a Ti 14 rotor using standard techniques (Reid [14], Bachofen, [15]). Details of the zonal separations are published elswhere [16].

The temperature of the gradient during the run was about 10 °C. The gradient consisted of (1) 275 ml of a slightly exponential gradient from 2.6–8.6% (w/v) sucrose, (2) a 50-ml band of 10.8% sucrose, and (3) 200 ml of a second exponential gradient from 12.6–14.4% and was followed by a 60% sucrose cushion. The gradient was buffered at pH 6.0 with 10 mM Tris–maleate and did not contain magnesium. The sample volume was 10–16 ml and the overlay 40 ml. After centrifugation at 45 000 rev./min ($\omega^2 \cdot t$ approx. $3.6 \cdot 10^{10}$ $rad^2 \cdot s^{-1}$) for 30 min, the gradient was removed from the rotor by displacement with 60% sucrose. It was collected in either (a) 5-ml

fractions, or (b) the following major fractions: the overlay and the first part of the gradient containing the unreacted $^{32}P_i$; band I containing the second part of the gradient, i.e., the ADP band in 10.8% sucrose; band II consisting of the total volume between band I and the chromatophore band, and finally the chromatophore band itself. This fractionation was checked following the radioactivity and the absorbance at 260 nm and 520 nm of the effluent.

Determination of the esterification capacity

Three 1.5-ml portions of the chromatophore band were incubated for 0 or 5 min in the dark at 30 °C in a mixture containing (total volume 4.5 ml): 5 mM $MgCl_2$, 0.1 mM sodium succinate, 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–NaOH (pH 8.0), 1.0 mg/ml serum albumin, and 2.5 mM ADP. The reaction was stopped by adding 2.5 ml of cold 1.0 M $HClO_4$. The suspension was frozen immediately in liquid air and stored at −20 °C until analysis.

The term "incubation" always refers to treatments carried out with preincubated and centrifuged chromatophores. The esterification capacity is defined as the increase of acid-soluble esterified phosphate during the incubation. It was determined either by the method of Avron [17] for total labeled esterified P_i or by quantitative DEAE-Sephadex A-25 column chromatography as described below. The total amount of P_i incorporated into PP_i, ADP and ATP found by chromatography was in good agreement with the esterified P_i according to Avron.

Solubilization of chromatophores and separation of proteins from low molecular weight components

Aliquots of the chromatophore band before and after the incubation period were mixed with equal volumes of 10% (w/w) $HClO_4$. After one min 11% (w/w) sodium dodecylsulfate was added to a final concentration of 1%. The mixture was allowed to stand for an additional minute and then brought to pH 3.0 with 2.5 M sodium acetate. The solution was immediately frozen in liquid air and stored at −20 °C. After adding marker nucleotides (1–3 μmoles each) proteins were separated from low molecular weight components at room temperature on a Sephadex G-50 column (35 cm · 2.5 cm) equilibrated with 0.1 M acetic acid containing 1% dodecylsulfate according to Mardh and Zetterquist [18]. The high molecular weight and the low molecular weight fractions were pooled separately. The fraction containing the total dodecylsulfate extractable nucleotides was either frozen and/or neutralized with K_2CO_3 to precipitate the $KClO_4$ and insolubilize the dodecylsulfate. After centrifugation the supernatant solution was decanted through a paper filter and prepared for chromatography on DEAE-Sephadex.

DEAE-Sephadex column chromatography

Chromatographic separations were performed on DEAE-Sephadex A-25 columns (20 cm · 1.5 cm) equilibrated with 0.1 M Tris–HCl buffer (pH 8.0). Samples for chromatography were diluted at least five times with Tris–HCl (pH 8.0) to a final buffer concentration of 50–75 mM. 1–3 μmoles of ATP, ADP, AMP, and PP_i were added as marker substances. The column was eluted with 500–1000 ml of a linear chloride gradient of 0–0.25 M NaCl in 0.1 M Tris–HCl, pH 8.0. The absorbance of the effluent was monitored continuously; preliminary measurements of the radio-

activity were performed with a methane flow counter. The different peaks were pooled according to their ultraviolet absorbance or their radioactivity (for P_i and PP_i).

Isotopic concentration experiments

Isotopic concentration experiments were performed in which carrier-free $^{32}P_i$ was added to the incubation mixture containing centrifuged chromatophores labeled with $^{32}P_i$ of a low specific activity (P*). The calculation of the concentration of inorganic phosphate and its specific activity were as indicated below. We had established from previous and these experiments that the specific activity (S_a) of the added $^{32}P_i$ was not changed by the endogenous inorganic phosphate. Assumed was (1) that the phosphate in the esterified compounds formed during preincubation had practically the same specific activity (S_a) as the $^{32}P_i$ (5 mM) added to the preincubation and (2) that during the incubation the added carrier-free $^{32}P_i$ of specific activity S_b equilibrated with the $^{32}P_i$ of specific activity S_a adsorbed to the centrifuged chromatophores. Phosphate which was esterified during preincubation exchanged with the added $^{32}P_i$ only as far as it was hydrolyzed during the 5-min incubation.

The final specific activity of $^{32}P_i$ (P**) in the incubation mixture of an isotopic concentration experiment, S_c, is

$$S_c = \frac{C_{high} - \Sigma c_{xv}}{\frac{C_{low} - \Sigma c_{xv}}{S_a} + m_b}$$

where

C_{low} = total cpm/ml in the incubation mixture of a usual preincubation experiment; c_{xv} = cpm/ml of esterified phosphate after the incubation in a usual preincubation experiment. Σ refers to the sum of counts measured in PP_i, ADP, and ATP; C_{high} = total cpm/ml in the incubation mixture of an isotopic concentration experiment; m_b = nmoles/ml of the added $^{32}P_i$ with a specific activity S_b

Radioactivity measurements

The radioactivity was measured in an aliquot of the preincubation mixture. of each of the bands or fractions resulting from centrifugation or chromatography either using a scintillation mixture (toluene, ethanol, PPO and POPOP) or diluted with water when using Cerenkov light [19]. Measurements were done in a Nuclear Chicago Model 724.

The color quenching by red chromatophores or solubilized chromatophores was corrected according to a channel ratio standard curve. All data were calculated in cpm incorporated on the day of the experiment using a half-life of 14.3 days for ^{32}P.

Chemicals

All chemicals were of the highest purity except the sucrose used in zonal gradients. ADP, ATP, and AMP were purchased from Boehringer, Mannheim, BRD. $^{32}P_i$ was obtained from Eidg. Institut für Reaktorforschung, Würenlingen, or from Radiochemical Centre, Amersham, England, and $^{32}PP_i$ from the latter firm.

RESULTS

Possible membrane-bound phosphorylated intermediates

Normal, partially uncoupled, and reconstituted chromatophores were preincubated with $^{32}P_i$ in the light or in the dark and then centrifuged on a gradient. The total esterification capacity of the centrifuged chromatophores is represented by the increase in acid-soluble labeled ATP, ADP and pyrophosphate during the subsequent dark incubation. The increase of acid-soluble PP_i is usually negligible and that of ADP is in the same range as that of ATP. The increase of acid-soluble ATP is defined as the partial esterification capacity and is given for some experimental conditions in Table I. Normal chromatophores preincubated in the dark show higher partial esterification capacities than those preincubated in the light. However, this capacity was light stimulated in uncoupled and reconstituted chromatophores.

On dodecylsulfate treatment additional acid-soluble nucleotides could be obtained from the chromatophores. This additional ATP, which may have been bound in some apolar or hydrophobic form to the membrane before the incubation with ADP, is also given in Table I. This firmly bound ATP (ATP_{fb}) could well be either the driving force or the source for the P_i transfer for the observed esterification capacity, provided that the amount of firmly bound ATP before the incubation is higher than the esterification capacity. Table I shows that this is the case. If the ATP_{fb} were

TABLE I

COMPARISON OF THE PARTIAL ESTERIFICATION CAPACITY AND THE AMOUNT OF FIRMLY BOUND ATP IN CHROMATOPHORES

The preincubation mixture contained in a total volume of 16 ml: 200 μg bacteriochlorophyll/ml, 5 mM KCl, 5 mM $MgCl_2$, 35 mM HEPES–NaOH (pH 8.0), 40 mM sucrose, 1 mg/ml serum albumin, 0.1 mM sodium succinate, 5 mM $^{32}P_i$ with a specific activity (S_a) on the day of the experiment of 6240 cpm/nmole P_i for chromatophores$_1$ and of 7320 cpm/nmole P_i for chromatophores$_2$. In the reconstituted system 1.02 mg protein/ml of a coupling factor preparation was added. AMP (10^{-4} M) was added where indicated. Chromatophores$_1$ were preincubated in the dark for 20 min (chromatophores$_2$ for 23 min) before and 5 min after the addition of $^{32}P_i$. A further dark or light preincubation lasted 3 min. During the first 23 min in the absence of added $^{32}P_i$ 1 min of light was given after the first 10 min in the chromatophores$_2$ experiments. n.d., not determined. Values in parentheses represent the change in firmly bound ATP during a dark incubation with ADP.

Preincubation	Phosphorylation activity (μmoles ATP/mg bacteriochlorophyll per h)	Partial esterification capacity (nmoles $^{32}P_i$ in ATP/mg bacteriochlorophyll)	Firmly-bound ATP before incubation (nmoles $^{32}P_i$ in ATP/mg bacteriochlorophyll)
Chromatophores$_1$ normal dark	0.4	2.8	4.9
Chromatophores$_1$ normal light	61.8	0.44	3.5
Chromatophores$_1$ uncoupled dark	0.7	0.1	0.6
Chromatophores$_1$ uncoupled light	11.7	0.25	1.0
Chromatophores$_1$ reconstituted light	36.5	0.6	2.2
Chromatophores$_2$ normal dark	0.4	0.4	5.1 (−4.4)
Chromatophores$_2$ normal light	26.0	0.2	1.3 (−0.2)
Chromatophores$_2$ normal light+AMP	n.d.	0.4	2.1 (+0.5)

to lead to an esterification of free ADP, the presence of ADP would induce a decrease in ATP_{fb}. This was tested by extracting the nucleotides before and after the incubation with either acid alone or acid in the presence of dodecylsulfate. The difference in the nucleotide analyses before and after the incubation (Table I, values in parentheses)

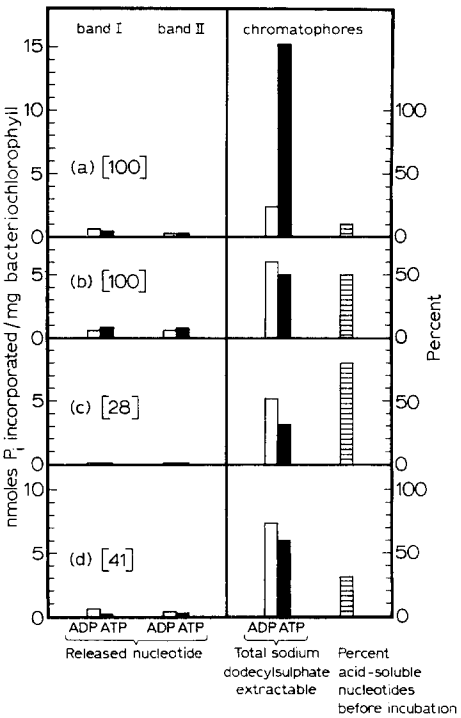

Fig. 1. Effect of ADP and cofactors, added as band I to the gradient in the zonal rotor, on the release of labeled nucleotides during centrifugation (bands I and II), on the amount of total dodecylsulfate-extractable nucleotides and on the acid-soluble nucleotides in the chromatophore band. Coupled and uncoupled chromatophores were preincubated in the dark for 10 min at 30 °C. Uncoupled chromatophores reconstituted by adding a coupling factor preparation were preincubated 20 min longer (in the absence of $^{32}P_i$). $^{32}P_i$ was added 2 min before "light on". The light lasted for 5 min in all cases. The preincubation mixture contained in a total volume of 16 ml: 250 μg bacteriochlorophyll/ml, 5 mM KCl, 10 $MgCl_2$, 37.5 mM HEPES–NaOH (pH 8.0), 50 mM sucrose, 1 mg/ml serum albumin, 0.1 mM sodium succinate and 5 mM $^{32}P_i$-sodium phosphate (pH 7.5–8.0) with a specific activity (S_a) on the day of the experiment of 18 860 cpm/nmole P_i. The reconstituted system also contained 1.25 mg protein/ml of a coupling factor preparation. The chromatophores moved through a sucrose gradient in which band I contained (where indicated) (total volume, 50 ml) 5 mM ADP, 10 mM HEPES–NaOH (pH 8.0) and 10 mM $MgCl_2$. The data given for the chromatophore band are calculated on the basis of the actual concentration of bacteriochlorophyll in the band. The results in bands I and II refer to the amount of bacteriochlorophyll added during the preincubation corrected for losses occurring during injection of the sample into the rotor. (a) Normal chromatophores after light preincubation, gradient without an ADP band, (b) normal chromatophores after light preincubation, gradient with an ADP band, (c) uncoupled chromatophores after light preincubation, gradient with an ADP band, (d) reconstituted chromatophores after light preincubation, gradient with an ADP band. Numbers in brackets refer to the percentage of coupling. Normal chromatophores showed a phosphorylation activity of 82.8 μmoles P_i esterified/mg bacteriochlorophyll per h.

represents the decrease in firmly bound ATP during the incubation period. The results show that firmly bound ATP indeed decreases enough during the incubation period to account for the observed esterification capacity. On the other hand, the extent to which acid-soluble ATP is formed (partial esterification capacity) compared to the decay of ATP_{fb} differs greatly between the light and dark experiments.

Evidence for a single one-step reaction

Experiments were done in which the chromatophores, loaded with ATP_{fb}, were centrifuged through a reaction band in the sucrose gradient containing ADP and Mg^{2+} [20]. These experiments demonstrated that the newly synthesized ATP remained bound to the chromatophores and did not exchange with free ADP in the band; it could, however, be liberated later by acid extraction of the chromatophore band.

Fig. 1 illustrates the amounts of labeled nucleotides released in the first two bands of the gradient as well as the nucleotides remaining in the chromatophore fraction. Graph a shows that preilluminated normal chromatophores lose only small amounts of labeled nucleotides when centrifuged in a gradient at pH 6.0 without added ADP nor Mg^{2+}. The addition of ADP and cofactors (graph b) does not significantly increase the release of labeled nucleotides but obviously changes the ratio of ATP to ADP which can be solubilized by dodecylsulfate from the chromatophores.

TABLE II

COMPARISON OF THE ESTERIFICATION CAPACITIES OF ISOTOPIC CONCENTRATION EXPERIMENTS WITH THOSE OF THE USUAL EXPERIMENTS

The conditions were given in Table I (Chromatophores$_1$) for the preincubation mixture. Those for the zonal gradient and the incubation of centrifuged chromatophores without additional $^{32}P_i$ were as given in Methods. In the isotopic concentration experiments the centrifuged chromatophores were incubated in the normal incubation mixture (given in Methods) supplemented with carrier-free $^{32}P_i$, approximately 100 μCi on the day of the experiment. The resulting specific activity of the inorganic phosphate was calculated for each type of centrifuged chromatophore as given in the Methods. The phosphate concentration was not adjusted by adding a known concentration of P_i in order to get as large differences as possible in the resulting specific activities (S_c/S_a) with the lowest change in the actual concentration of inorganic phosphate. Indices h and l refer to the isotopic concentration experiments and to the usual experiments, respectively.

Preincubation	Ratio of specific activities of P_i S_c/S_a	Labeling ratio in ATP and ADP		Carrier-free $^{32}P_i$ as a percentage of total P_i in the isotopic concentration experiments during incubation
		$\frac{ATP^h_{as}}{ATP^l_{as}}$	$\frac{ADP^h_{as}}{ADP^l_{as}}$	
Chromatophores normal dark	14	13	7	2
Chromatophores normal light	186	132	6	33
Chromatophores uncoupled dark	41	19	1.2	8
Chromatophores uncoupled light	18	17	0.8	3
Chromatophores reconstituted light	18	17.5	–	3

More important, ADP reduced the dodecylsulfate-extractable ATP_{fb} and increased the portion of acid-soluble nucleotides (last column Fig. 1). The ATP_{as} thus formed was soluble in acid but not in buffer; that is, it was not released from or washed off the chromatophores after passing the ADP band, but had to be liberated from the chromatophore band by an acid extraction. This would indicate that a single one-step reaction had taken place while the chromatophores moved through the ADP band.

Graphs c and d show that there is a correlation between the degree of coupling and both the release of nucleotides into bands I and II and the amount of extractable labeled nucleotides in the chromatophore band.

The experiments described suggest a mechanism of phosphorylation in which the formation of acid-soluble ATP is driven by the hydrolysis of firmly bound ATP presumably bound to the coupling factor.

Evidence against a direct γ-phosphate transfer

Table II illustrates the results obtained in an isotopic concentration experiment. It shows that the ATP^{h}_{as} synthesized during the incubation of prelabeled P* chromatophores with P_i^{**} and ADP had the specific activity corresponding to ATP** and not to ATP*. This excludes the possibility of a direct γ-P transfer from ATP_{fb} to ATP_{as}. Fig. 2 shows besides a direct transfer of γ-P (1), three possible reaction sequences involving free phosphate. In Case 2, the mechanism is essentially comparable to that in Case 1 except that the γ-P of ATP_{fb} can exchange with P** to a certain extent. In Case 3 the hydrolysis of ATP_{fb} supplies the energy which is transduced by a coupling

Proposed mechanisms	Isotopic concentration experiment P* → P**		Usual experiment P*	Labelling ratio in ATP	Labelling ratio in ADP
	ATP^{*}_{as}	ATP^{**}_{as}	ATP^{*}_{as}	$\frac{ATP^{h}_{as}}{ATP^{l}_{as}}$	$\frac{ADP^{h}_{as}}{ADP^{l}_{as}}$
1) $ADP \sim P^{*}_{fb} + P^{**} \rightarrow ADP + P^{**}$	$a\,S_a$	0	$a\,S_a$	1	1
2) $ADP \sim P^{*}_{fb} + P^{**} \rightarrow ADP \sim P^{**}_{fb} + P^{*}$	$ax\,S_a$	$a(1-x)S_c$	$a\,S_a$	$x + (1-x)\frac{S_c}{S_a}$	1
3) $ADP \sim P^{*}_{fb} \rightarrow ADP$; $ADP_s + P^{**} \rightarrow ATP^{**}_{as}$	0	$a\,S_c$	$a\,S_a$	$\frac{S_c}{S_a}$	
4) $2\,ADP_s \rightarrow ATP_s + AMP_s$; $ATP_s + P^{**} \rightarrow ATP^{**}_{s} + P$; $ATP^{**}_{s} + AMP_s \rightarrow ADP^{**}_{s} + ADP$	0	$a\,S_c$	$a\,S_a$	$\frac{S_c}{S_a}$	$1 < y < \frac{S_c}{S_a}$

Fig. 2. Possible mechanisms of the energy transduction responsible for the esterification capacity and the expected experimental results. ADP ~ P_{fb} refers to firmly-bound ATP labeled during the preincubation with P_i of specific activity S_a. P** stands for the P_i of the specific activity of the inorganic phosphate in an isotopic concentration experiment, S_c as calculated in the Methods. Subscripts s and as mean soluble and acid-soluble, respectively. "a" represents a certain amount of nmoles P_i incorporated into ATP in a usual experiment. Further explanations in the text.

factor for the synthesis of a new ester bond between added ADP and P**. Mechanism 4 is dependent on the presence of a functional adenylate kinase in the centrifuged chromatophores and does not involve ATP_{fb}. In this scheme ATP produced from added ADP by the adenylate kinase takes part in a classical ATP–$^{32}P_i$ exchange reaction.

The labeling patterns and the expected labelling ratios of radioactive acid-soluble ATP from an isotopic concentration experiment allowed us to differentiate between these mechanisms. The experimentally obtained labeling ratios of acid-soluble ATP (Table II) approximate the specific activity ratios of the inorganic phosphates (S_c/S_a) except where the addition of carrier-free phosphate changed the phosphate concentration more than 5% (Table II, last column). Mechanism 2 could only give the same labeling ratios (S_c/S_a) as 3 if the P_i transfer from ATP_{fb} to added ADP were slower than the exchange reaction. This is improbable since photophosphorylation rates have been shown to be either equal or over 10 times higher than the rates of the exchange reaction [21, 22]. If the esterification capacity were due to Mechanism 4 which requires the participation of an adenylate kinase [23], the free ATP** produced in an isotopic concentration experiment would lead to a lower but comparable increase in the label (ADP**) of the ADP^h_{as}. Table II shows that this is not the case, except perhaps for the experiment with normal chromatophores. Although the labeling ratio of ADP for normal chromatophores after light preincubation differs from one, it is much closer to one than to S_c/S_a. It also is of approximately the same proportion to its S_c/S_a value, that is 6/186, as for uncoupled chromatophores, 1.2/41.

The labeling ratio of ADP could imply that labeled acid-soluble ADP appearing during the incubation does not have to originate from a simultaneous action of adenylate kinase, but could represent the ADP liberated during the breakdown of double-labeled firmly bound ATP. Dodecylsulfate-extracted ATP obtained from chromatophores which were illuminated during the preincubation before the addition of $^{32}P_i$ was equally labeled in the β- and γ-P. This was determined by incubating the isolated ATP with hexokinase and glucose. The ADP thus obtained contained 48% of the total radioactivity previously found in ATP.

Contrary to the above findings with light preincubated normal, uncoupled and reconstituted chromatophores, normal dark preincubated chromatophores or light preincubated chromatophores supplemented with 10^{-4} M AMP (not shown) exhibit an ADP labeling ratio which follows that of ATP. This suggests that an adenylate kinase and an ATP–$^{32}P_i$ exchange (Mechanism 4) are responsible for the labeling of the nucleotides during the incubation in these assays. In the presence of AMP an increase of ATP_{fb} can be seen (Table I) which correlates with the increase of the labeling ratio of ADP and in addition supports the possible involvement of Mechanism 4 under these conditions.

DISCUSSION

Hypotheses concerning the mechanism of the last step of phosphorylation in energy transducting membranes include among others a direct transfer of P_i bound to the coupling factor to the added ADP [2, 24, 25]. Elimination of Mechanisms 1, 2, and 4 as discussed under the Results leaves us with Mechanism 3 as the most probable. We have shown in Fig. 1 that the ATP_{as}, which arises when ATP_{fb}-rich chromatophores pass through an ADP band in the centrifuge rotor, is not released.

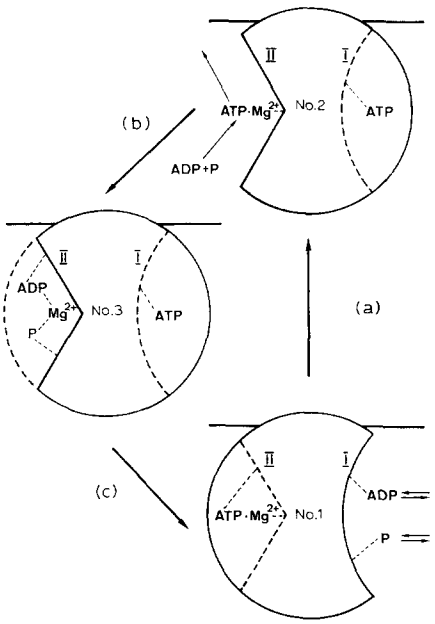

Fig. 3. Scheme concerning the hypothesis of the last steps of photophosphorylation in *R. rubrum*. Process (a) the light-driven electron transport energizes the coupling factor and thus leads to a phosphorylation of adsorbed ADP and P_i to "hydrophobically bound" ATP at Site I and makes Site II accessible to water. (b) Previously bound ATP at Site II exchanges with ADP and P_i and thus reaches a metastable state. (c) Site I de-energizes providing Site II with the necessary conformational change needed to favor a phosphorylation to ATP. (Further explanations in the text).

This finding and the evidence in favor of Mechanism 3 suggests that the coupling factor involved has at least two sites, possibly with different and changing conformations, one for ATP_{fb} and another for ATP_{as}. The possibility of two sites which seem to differ in their surroundings and in Mg^{2+} requirements is also suggested by the fact that ATP_{fb} does not need Mg^{2+} for stability during centrifugation. Furthermore, phosphate binding to twice-washed chromatophores in the absence of Mg^{2+} is stimulated when the chromatophores are preincubated in the light with low amounts of ADP (Suter, W., Lutz, H. U. and Bachofen, R., unpublished).

A formulation which would satisfy the above mentioned requirements is illustrated in Fig. 3. It basically states that the energized state of the coupling enzyme, induced by a proton flux [Mitchell, 26] or by a change in the redox state of an electron carrier [27], corresponds to a hydrophobic environment (Site I) in which the equilibrium between ATP and the adsorbed ADP and free phosphate is displaced in favor of ATP_{fb} (transition from diagram 1 to 2 in Fig. 3). Site II now becomes accessible to water and the ATP_{as} formed during one revolution of the cycle can exchange with ADP and phosphate; the ATP is released as soluble ATP, and ADP, phosphate, and Mg^{2+} adsorb again to Site II (diagram 3 in Fig. 3). New ATP_{as} is now formed at Site II with the concomitant hydrolysis of ATP_{fb} at Site I. The energy content of Site I has been transferred to Site II, and Site I now becomes accessible to water. This water will be excluded when the coupling factor is re-energized by the electron transport

chain. The proposed mechanism can explain both the ATP–$^{32}P_i$ and the ATP–[^{14}C]-ADP exchange reactions.

Recent papers give some support to our working hypothesis. Yamamoto et al. [28] have shown that in *R. rubrum* bound ADP is phosphorylated to bound ATP prior to the detection of free ATP. Cross and Boyer [29] and Boyer et al. [30] have presented evidence for bound ATP as an intermediate in mitochondrial oxidative phosphorylation. Consistent with our results in *R. rubrum* they found that the enzyme-bound ATP was precipitable by $HClO_4$ and not covalently bound to proteins, in contrast with their previous findings [31]. The involvement of bound ATP in the last step of energy transduction has also been suggested by the inhibition of phosphorylation by ATP (Shavit [32] and Walz et al. [33]).

Publications by Fisher et al. [34], Ryrie and Jagendorf [35], Harris et al. [36], Murakami and Packer [37], Williams [38], and others support a phosphorylation mechanism involving conformational changes of the coupling enzyme or factor. Our hypothesis not only is compatible with this concept but requires it.

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