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BOUND NUCLEOTIDES AND PHOSPHORYLATION IN RHODOSPIRILLUM RUBRUM

David A. Harris* and Margareta Baltscheffsky
Arrhenius Laboratory, Department of Biochemistry,
University of Stockholm, S-106 91 Stockholm, Sweden

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SUMMARY. Chromatophores from Rhodospirillum rubrum contain 12 x 10^{-3} mol ATP and 8.3 x 10^{-3} mol ADP per mol chlorophyll, tightly bound to the coupling ATPase. Under energised conditions, these exchange slowly with added nucleotide. Using single turnover light flashes, it is demonstrated that the release of bound ATP is too slow to be on the direct pathway of photophosphorylation.

INTRODUCTION. Coupling ATPases from a wide variety of organisms bear tightly bound ATP (2 mol/mol ATPase) and ADP (1mol/mol ATPase). (For a review see [1]). In 1973, Harris et al [2] suggested that these nucleotides were involved directly in the mechanism of ATP synthesis. The essence of this hypothesis was:

i) ATP synthesis on the ATPase required no energy, the energy-requiring step in phosphorylation being the release of ATP from the enzyme, and

ii) The tightly bound ATP represented a (low-energy) intermediate in phosphorylation, and was thus bound at an active site in one of its states. It should be pointed out that no proposals were made as to the role of the bound ADP.

The first postulate, of a nil energy requirement for ADP-P_i bond formation, is supported by work on the P_i - $H_2{}^{18}O$ exchange reaction [3] and on NTP-driven reactions in submitochondrial particles [4].

In support of the second postulate, it was shown that the bound nucleotide would exchange with added nucleotide only under energised conditions [5-7, see also 1]. However, the time resolution of these experiments was too low to allow assignment of the bound ATP to the mechanism of phosphorylation. Attempts

* Present Address. Department of Biochemistry, University of Leeds, 9 Hyde Terrace, Leeds, Yorkshire, U.K.

Abbreviations: NTP, NDP, nucleoside tri, diphosphate. rro-ATP (ribose-ring opened ATP) 2-2 [1-(9-adenyl)-1'-triphosphoryl-oxymethyl)-dihydroxydiethyl ether.

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to increase the resolution [8-10] foundered, in chloroplasts, on the presence of an inhibitor protein which limits phosphorylation [10], allowing only a small fraction of ATP synthase molecules to be active during short flashes [9,10]. Kinetic studies on incorporation of ^{32}P into the bound ATP of E. coli cells [11] and submitochondrial particles [12] have been taken to imply that bound ATP is not an intermediate in phosphorylation. However, these studies also could not show what fraction of ATPase molecules were turning over in the time of measurement, and thus it is not valid to draw such a conclusion. We have indeed shown that, in submitochondrial particles as in chloroplasts, the number of active ATPase molecules is limited by the inhibitor protein ([10], Harris, D.A., van Tscharner, V. and Radda, G.K., submitted for publication).

In R. rubrum chromatophores, the ATPase inhibitor protein is largely absent or inactive - e.g. the dark hydrolysés rate is of a similar order to the phosphorylation rate. A single turnover of the respiratory chain (by a 10 μs light flash) would thus be expected to cause a single turnover of all coupled ATP synthase molecules present. Using luciferase to measure the ATP produced, a single turnover flash can indeed be shown to lead to ATP production corresponding to turnover of a substantial fraction of ATPase molecules ([13] below) in R. rubrum, in contrast to the situation in chloroplasts [10].

Since purified luciferase is very specific for adenine nucleotides [14], if, say, dADP is used as a substrate for phosphorylation, the source of the first NTP formed - whether from bound ATP or newly phosphorylated dADP - can be determined. It is shown below that, during one or two turnovers of the respiratory chain, bound ATP was not released while free dADP, IDP or GDP were indeed phosphorylated. It was concluded that tightly bound ATP does not occupy an active site for phosphorylation.

MATERIALS AND METHODS R. rubrum chromatophores were prepared by the method of Baltscheffsky [15]. Total phosphorylation was measured using a sensitive pH electrode, by the method of Nishimura et al [16]. Phosphorylation of ADP by the chromatophores was typically 8-9 molATP/mol chlorophyll/min under saturating light.
Luciferase, purified by the method of Lundin [17] and luciferin were kindly supplied by Dr. A. Lundin. Luminescence was measured in an apparatus constructed

by B. Höijer in this laboratory. Actinic illumination was obtained via a light guide provided with double layers of Wratten 88A gelatin filter, and the photomultiplier tube was protected from scattered light with a Corning 9782 glass filter.
Nucleotides were obtained as described elsewhere [4]. None of the triphosphates of those used here gave more than 5% of the luminescence observed with ATP at comparable concentrations in the luciferase assay (not shown). The bound nucleotide content of the chromatophore membrane was measured essentially as described by Harris and Slater [5].

RESULTS AND DISCUSSION. Bound nucleotides in R. rubrum.

Table I shows the amount of ATP and ADP tightly bound in R.rubrum chromatophores (as extracted by 4% perchloric acid). These values correspond to a value of 1 - 2 mol ATP per mol ATPase if the amount of ATPase is estimated by the method of Ferguson et al. [18] (Harris, D.A. and Baltscheffsky, M., unpublished), or is assumed to correspond to the number of antimycin binding sites [19]. Also in agreement with other systems [1], the ATP/ADP ratio is close to 1.5.

The bound nucleotides exchange to a limited extent with added [^{14}C]ATP when the membrane is energised (Table I). dATP also seems to be able to displace adenine nucleotides, and, in particular, we see that the bound ADP is much more susceptible to displacement.

Table I also shows that, when washed particles are illuminated in the absence of added nucleotide, there is a slow release of ATP to the solution. This must represent release of bound ATP (or phosphorylation and release of bound ADP) since no other nucleotides are present. The release is slow ($t_{\frac{1}{2}}$ = 3s), and incomplete - presumably because it represents a competition between the ATPase and luciferase for the released ATP.

Nucleotide specificity of coupled processes.

Table II shows that, in contrast to submitochondrial particles [4], chromatophores have a wide nucleotide specificity of (coupled) phosphorylation and NTP-driven reversal of electron transfer, as well as of hydrolysis. In part this may be due to the very active NDP-kinase activity in these particles. However, evidence is presented below that dADP, IDP and GDP, at least, are phosphorylated on the coupling ATPase directly.

TABLE I. Bound nucleotides in R. rubrum chromatophores

	ATP bound	ADP bound	[^{14}C]nucleotide bound	ATP released on continuous illumination
	(mmol per mol bacteriochlorophyll)			
[^{14}C]ATP	12	8.3	4.8	2.2
dATP	10	2.3	-	1.0

Chromatophores were labelled with 200 μM [^{14}C]ATP under illumination as described [5] except that the buffer used contained 200mM glycylglycine, 5mM NaP_i, 2mM $MgCl_2$ and 0.1mM succinate (pH 7.4 with NaOH). Treatment with dATP was carried out similarly, with 200μM dATP replacing [^{14}C]ATP. Bound nucleotides were assayed, with luciferase, as described [5].

ATP release was measured by illuminating chromatophores with saturating, continuous light in the presence of luciferase + luciferin in the photometer described in 'Methods'.

Control particles (prepared without preincubation) contained bound ATP and ADP identical in amount (within experimental error) to particles pre-incubated with [^{14}C]ATP. Release of ATP on illumination was also identical (not shown).

TABLE II. Nucleotide specificity of coupled processes in R. rubrum

Nucleoside base	Phosphorylation	NAD^+ reduction	Hydrolysis
adenosine	100[a]	100[b]	100[c]
deoxyadenosine	69	68	72
inosine	46	43	76
guanosine	49	53	56
N^1-oxido adenosine	24	<5	33
iso-guanosine	18	34	not determined
rro-adenosine	<5	<5	<5
adenosine (no P_i)	<2	-	-

NAD^+ reduction by succinate was measured by the method of Keister and Yike [20] and NTP hydrolysis, under identical conditions, by monitoring P_i release [21]. NDP concentrations for phosphorylation ranged up to 400μM, and NTP concentrations for hydrolysis and NAD^+ reduction up to 3mM. The maximum values (close to V_{max}) are given in all cases.

[a] = 6.7 mol ATP/mol chlorophyll/min
[b] = 0.24 mol NADH/mol chlorophyll/min
[c] = 2.5 mol P_i/mol chlorophyll/min

ATP release during flash-induced phosphorylation

Table III shows the amount of ATP formed in solution after a 1ms flash. If saturating ADP is present, about 5mmol free ATP/mol chlorophyll (0.5-1mol/mol ATPase) is released, in agreement with [13]. A shorter flash - one turnover of the respiratory chain - yields half this amount of ATP. It was concluded that,

TABLE III Flash yield of ATP by chromatophores

Nucleotide	Free ATP after flash. (mmol/mol chlorophyll) 10 μs flash	1 ms flash
none	-	0.14 (± 0.04)
ADP (25μM)	2.26 (± 0.2)	4.56 (± 0.4)
dADP (150μM)	-	0.05 (± 0.03)
IDP (600μM)	-	<0.2
GDP (250μM)	-	<0.2
N^1-oxido ADP (200μM)	-	<0.2
rro-ADP (200μM)	-	<0.2
8-bromo ADP (200μM)	-	<0.2
ADP (25μM) (no P_i)	1.17 (± 0.18)	1.16 (± 0.10)

ATP release following a 10μs (1 turnover) and 1ms (2 turnover) flash was measured as previously [13], except that other nucleotides replaced ADP as indicated. The error represents the range of 4 duplicate readings. 4mM P_i was present except where indicated.

in this preparation, a single turnover of the respiratory chain leads to one turnover of a significant (measurable) fraction of ATP synthase molecules, unlike in chloroplasts [10], presumably because of the lack of inhibitor protein in chromatophores. It should be noted that in this system, a 10μs flash causes one turnover of the respiratory chain, and a 1 ms flash, on average, two turnovers. This situation arises because the step rate limiting to the second turnover is re-reduction of the chlorophyll after the initial (fast) photooxidation.

Analogues like dADP, IDP and GDP, which are phosphorylated (Table I) yield virtually no free ATP. If bound ATP were an intermediate in phosphorylation, the first turnovers of the ATPase would yield free ATP even when the analogues were being phosphorylated. This clearly does not occur.

To counter this argument, we might consider that dADP, IDP and GDP are, for some reason, not phosphorylated during the first couple of turnovers, or even that their phosphorylation does not directly involve the coupling ATPase but, say, NDP kinase. To test these possibilities, the effect of the nucleotides on ADP phosphorylation was tested. In either case, we would expect the inhibition by these nucleotides on ADP phosphorylation (ATP release) to be absent in short flashes since they are not 'seen' by the coupling ATPase. They would, however, inhibit ATP production in continuous light either by occupying the coupling ATPase or by using the terminal phosphate of newly produced ATP.

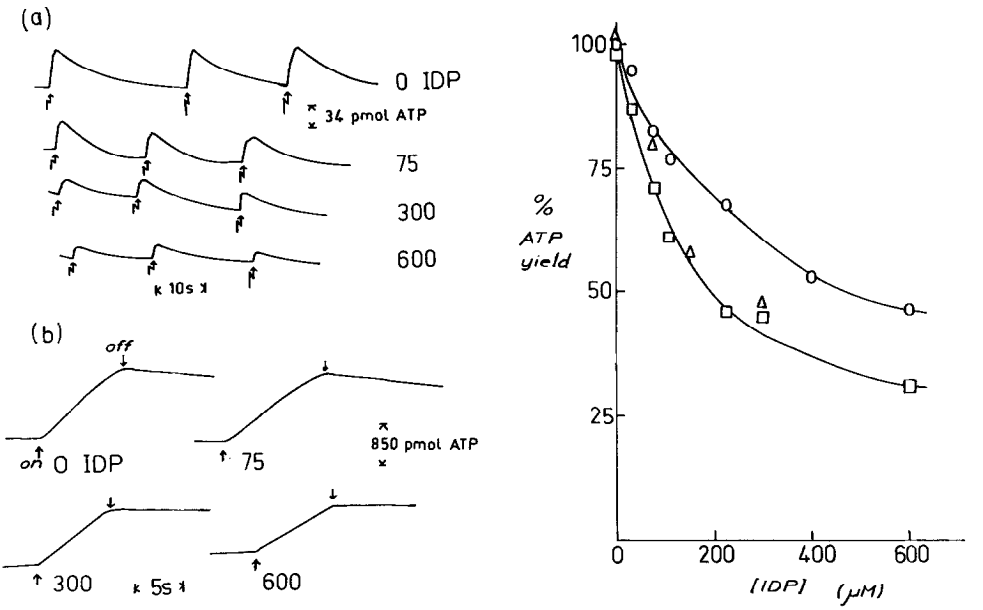

Fig.1. Inhibition of ADP phosphorylation by IDP. Flash induced phosphorylation in the presence of 2.5μM ADP and various amounts of IDP was measured as in Table III. ADP phosphorylation in continuous light was measured in the same apparatus. 100% represents 2.5 mol ATP/mol chlorophyll/min in the case of continuous light phosphorylation, 0.6mmol ATP/mol chlorophyll/10μs flash and 2.26mmol ATP/mol chlorophyll/1ms flash.

Typical traces are shown (left) for a 1ms flash (a) and for continuous light phosphorylation (b).

Δ—Δ 10μs flash, □—□ 1ms flash, O—O continuous light.

The luciferase assay provides a simple method for measuring ADP phosphorylation in the presence of the phosphorylation of other nucleotides owing to its high specificity for ATP [14] Fig. 1 shows the inhibition of flash-induced, and continuous light induced production of ATP at non-saturating ADP concentrations, in the presence of IDP. It is clear that ATP production by single or double turnover flashes is even more strongly inhibited by IDP than ADP phosphorylation under continuous light. (The difference in K_i in the two cases probably results from the different energy states of the membrane in flash and continuous light). dADP and GDP behave similarly. The corresponding $c_{\frac{1}{2}}$ (concentration for 50% inhibition) values for dADP are 6 μM (flash) and 25 μM (continuous light), and for GDP, 160 μM (flash).

We conclude, therefore, that IDP, dADP and GDP are phosphorylated by the coupling ATPase directly, and during its first turnover(s). This conclusion,

and the finding that ATP is not released from the coupling ATPase under these conditions, rules out the possibility that tightly bound ATP is an intermediate in phosphorylation. This is directly opposed to the models of Harris et al. [2,5] and Koslov and Skulachev [22]. These findings also confirm that bound ADP is not the initial phosphate acceptor in phosphorylation [24].

Flash-induced production of free ATP, with ADP but no added P_i (Table III), i.e. under conditions of no net phosphorylation (Table II)-is interesting. In this case, involvement of bound ATP [1] or of bound P_i [23] is suggested - probably the latter since 5mM arsenate inhibits this ATP production by 75% (not shown). That some bound species is involved is supported by the finding that a second turnover of the respiratory chain does not increase the ATP yield above the single turnover yield (Table III) in contrast to the situation where P_i is present.

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REFERENCES

1. Harris, D.A. (1978) Biochim. Biophys. Acta 463, 245-273
2. Harris, D.A., Rosing, J., van de Stadt, R.J. and Slater, E.C. (1973) Biochim.Biophys.Acta 314, 149-153
3. Boyer, P.D., Cross, R.L. and Momsen, W. (1973) Proc. Nat. Acad. Sci. (U.S.) 70, 2837-2839
4. Harris, D.A., Gomez-Fernandez, J.C., Klungsøyr, L. and Radda, G.K. (1978) Biochim. Biophys. Acta in press.
5. Harris, D.A. and Slater, E.C. (1975) Biochim. Biophys. Acta 387, 335-348
6. Strotmann, H., Bickel, S. and Huchzermeyer, B. (1976) FEBS Lett. 61, 194-198
7. Magnusson, R.P. and McCarty, R.E. (1976) Biochem. Biophys. Res. Commun. 70, 1283-1289
8. O'Keefe, D. and Dilley, R.A. (1977) FEBS Lett. 81, 105-110
9. Gräber, P., Schlodder, E. and Witt, H. (1977) Biochim. Biophys. Acta 461, 426-440
10. Harris, D.A. and Crofts, A.R. (1978) Biochim. Biophys. Acta 502, 87-102
11. Maeda, M., Kobayashi, H., Futai, M. and Anraku, Y. (1977) J. Biochem. 82, 311-314
12. Boyer, P.D., Gresser, M., Vinkler, C., Hackney, D. and Choate, G. (1977) in 'Structure & Function of Energy-Transducing Membranes' (van Dam, K. and van Gelder, B.F. eds.) pp261-274 Elsevier, North Holland, Amsterdam
13. Lundin, A., Thore, A. and Baltscheffsky, M. FEBS Lett. 79, 73-76
14. Lee, R.T., Denburg, J.L. and McElroy, W.D. (1970) Arch. Biochem. Biophys. 141, 38-52
15. Baltscheffsky, M. (1967) Nature 216, 241-243
16. Nishimura, M., Ito, T. and Chance, B. (1962) Biochim. Biophys. Acta 59, 177-182
17. Lundin, A. (1978) Methods in Enzymology Vol. 57 (deLuca, M. ed) pp.56-65 Academic Press, New York

18. Ferguson, S.J., Lloyd, W.J. and Radda, G.K. (1976) Biochem. J. 159, 347-353
19. Yamamoto, N., Yoshimura, S., Higati, T., and Horio, T. (1972) J. Biochem. 72, 1397-1406
20. Keister, O.L. and Yike, N.J. (1967) Arch. Biochem. Biophys 121, 415-422
21. Rathburn, W.B. and Betlach, M.V. (1969) Analyt. Biochem. 28, 436-446
22. Koslov, I. and Skulachev, V.P. (1977) Biochim. Biophys. Acta 463, 29-89
23. Suter, W., Lutz, H.U. and Bachofen, R. (1976) Eur. J. Biochem. 65, 57-60
24. Rosing, J., Smith, D.J., Kayalar, C. and Boyer, P.D. (1976) Biochem. Biophys. Res. Commun. 72, 1-8