BACTERIAL PHOTOPHOSPHORYLATION IN THE ABSENCE OF ADDED NUCLEOTIDE. A SECOND INTERMEDIATE STAGE OF ENERGY TRANSFER IN LIGHT-INDUCED FORMATION OF ATP

Herrick Baltscheffsky and Lars-Victor von Stedingk

Wenner-Gren Institute and Department of Biochemistry, University of Stockholm, Stockholm, Sweden.

Received February 18, 1966

Two properties of isolated chromatophores from Rhodospirillum rubrum make this photosynthetic system particularly suitable for studies of certain mechanisms involved in the light-induced energy transfer which is coupled to cyclic electron transport and leading to formation of ATP. Firstly, in contrast to isolated chloroplasts from green plants the bacterial chromatophores contain a cyclic photophosphorylation system functioning at high rates even in absence of any added electron carrier. Secondly, the photosynthetic system of the bacterial chromatophores shows a number of striking similarities to the mitochondrial oxidative phosphorylation system of cellular respiration with respect to sensitivity towards some specific inhibitors of both electron transport and electron transport-coupled energy transfer reactions, such as HOQNO, antimycin A, valinomycin, gramicidin, and oligomycin.

A light-induced, rapidly reversible disappearance of inorganic orthophosphate was found to occur when chromatophores from Rhodospirillum rubrum were illuminated in the absence of added adenosine nucleotide (Horio, von Stedingk and Baltscheffsky, 1966). A possible explanation for this disappearance could have been esterification with bound adenine nucleotide. However, a detailed analysis of the product formed was required. In this paper we present some new data allowing identification of the apparent location of the phosphorylated product, which is inorganic pyrophosphate (Balt-

Abbreviations: HOQNO, 2-heptyl-4-hydroxyquinoline-N-oxide; PMS, phenazine methosulfate; PP, inorganic pyrophosphate.

scheffsky, von Stedingk, Heldt and Klingenberg, in preparation) and seems to be in rapid equilibrium with the well-known but still hypothetical phosphorylated intermediate X~P of various schemes for electron transport-coupled energy transfer. Pyrophosphate formation accordingly represents a second intermediate stage connected with the energy transfer system between electron transport and ATP in this bacterial photophosphorylation, the first stage being the light-induced, reversible pH-change obtained in the absence of added orthophosphate, ADP, and $MgCl_2$ (von Stedingk, in preparation; Baltscheffsky and von Stedingk, 1966).

EXPERIMENTAL. The bacteria were grown and harvested, the chromatophores were prepared, the reactions were performed and the material subsequently analyzed, unless otherwise mentioned, as reported by Horio, von Stedingk and Baltscheffsky (1966).

Table I shows the data from an experiment which was made in order to determine whether pyrophosphate formation in absence of added adenine nucleo-

Table I.

Inhibition of light-induced phosphate uptake in absence of added ADP by HOQNO and restoration by PMS.

Reaction medium: 1.5 ml total volume containing 0.1 ml 0.1 M $NaH_2{}^{32}PO_4$, 0.1 ml 0.2 M $MgCl_2$, 0.1 ml $1.5 \cdot 10^{-2}$ M Na-ascorbate, 0.5 ml 0.2 M glycylglycine-NaOH buffer pH 7.4, 0.1 ml chromatophore suspension giving a final O.D.$_{880\ m\mu}$ of 4.3. This mixture was preincubated in the dark for 4 min. before illumination (about 30.000 lux). The low incorporation of $^{32}P_i$ during this dark period was taken as zero and thus subtracted from the amount of $^{32}P_i$ incorporated during the reaction in light and darkness. The reaction was stopped by addition of 1 ml cold 2 M perchloric acid. Reaction time 40 sec.

Additions	Phosphate uptake in m moles P_i/O.D.$_{880\ m\mu}$	Per cent of initial value
-	6.5	100
$5 \cdot 10^{-6}$ M HOQNO	0.3	5
$5 \cdot 10^{-6}$ M HOQNO + $1.2 \cdot 10^{-4}$ M PMS	7.6	116

tide was linked only to the "physiological system" for cyclic photophosphorylation, as was the light-induced pH-change (Baltscheffsky and von Stedingk, 1966), or also to the "PMS-system" (these systems were described by Baltscheffsky, 1960). It is seen that HOQNO inhibits the disappearance of orthophosphate in the "physiological system" and that this inhibition is overcome when the electron carrier PMS is present. Light-induced formation of pyrophosphate in absence of added nucleotide is thus linked not only to the "physiological system" but also to the "PMS-system" and differs in this respect from the light-induced pH-change but is similar to photophosphorylation of added ADP.

Oligomycin inhibits energy transfer in mitochondrial oxidative phosphorylation at a site closely connected with the final ATP-forming reaction. This characteristic of oligomycin, when taken together with its strong inhibitory effect on photophosphorylation of added ADP in the bacterial chromatophores (H. Baltscheffsky and M. Baltscheffsky, 1960), makes it a powerful tool for gross localization of a phosphorylated product in the bacterial light-induced energy transfer system. If oligomycin inhibits light-induced formation of a phosphorylated product in absence of added nucleotide, then the final step in the formation of that product would be more distant from the electron transport chain than the site of action of oligomycin. On the other hand, lack of effect or stimulation would indicate that the reaction product either is an intermediate in the energy transfer pathway between the electron transport chain and the site of action of oligomycin, or is emerging from one. Fig. 1 shows that oligomycin slightly <u>stimulates</u> light-induced phosphate disappearance in absence of added nucleotide. As the initial rate of phosphate disappearance in absence of added nucleotide is very low as compared to the over-all rate of ATP-formation with added ADP the product formed is on a side-path from an intermediate of the energy transfer pathway leading to ATP and not an actual intermediate (Fig. 2). The final product obtained under these conditions is not absorbed on charcoal in acid solution. By gradient elution chromatography

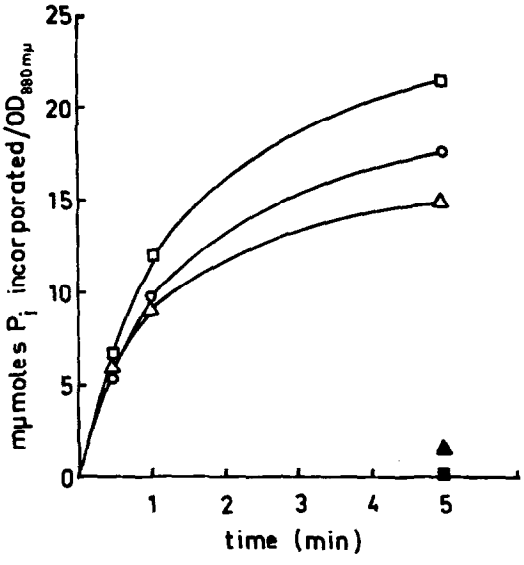

Fig. 1. Effect of oligomycin on light-induced phosphate uptake in absence of added ADP. Experimental conditions as in Table I. Triangles: no addition; circles: 1 μg oligomycin; squares: 10 μg oligomycin. Open and solid symbols show the reactions in light and darkness, respectively.

---

on Dowex 2-X 10 (Lindenbaum, Peters and Rieman, 1954; Grande and Beukenkamp, 1956) it was shown to be inorganic pyrophosphate. For example, during a 6 min. illumination period of a chromatophore suspension of $O.D._{880 mμ} = 12.5$, in presence of 20 μg oligomycin/3.2 ml total volume, 195 mμmoles of P_i disappeared as measured by extraction of bound phosphate from inorganic orthophosphate according to the method of Avron (1960), and by chromatography 184

Table II

Effects of gramicidin and valinomycin on light-induced phosphate uptake in absence of added ADP.

Experimental conditions as in Table I.

Additions	Phosphate uptake in mμmoles $P_i/O.D._{880 mμ}$		
	30 sec.	60 sec.	5 min.
-	5.8	9.0	15.0
2.10^{-6} M gramicidin + 15 mM KCl	0.9	1.5	1.2
-	2.9	5.5	9.2
3.10^{-6} M valinomycin + 15 mM KCl	3.3	4.8	9.9

mμmoles were recovered in the pyrophosphate fractions, which were completely separated from the fractions containing the orthophosphate and tripolyphosphate markers. This difference is within the experimental error of the method. The dark control contained negligible amounts of pyrophosphate.

This low rate of the final reaction(s) of photophosphorylation in the absence of added nucleotide appears to provide the explanation of the results obtained with valinomycin, which only partially inhibits photophosphorylation of added ADP (Baltscheffsky and Arwidsson, 1962) and gramicidin, which virtually completely inhibits this photophosphorylation (H. Baltscheffsky and M. Baltscheffsky, 1960). As Table II shows, valinomycin is not capable of inhibiting light-induced phosphate disappearance in absence of added nucleotide, whereas gramicidin is. Inhibition of the comparatively slow final reaction(s) of pyrophosphate formation appears to require agents which very strongly inhibit the much more rapid initial energy transferring reactions of the system.

DISCUSSION. The connection between the reactions leading to formation of pyrophosphate in absence of added nucleotide and the energy transfer pathway for light-induced ATP-formation is given in Fig. 2, which for the sake of

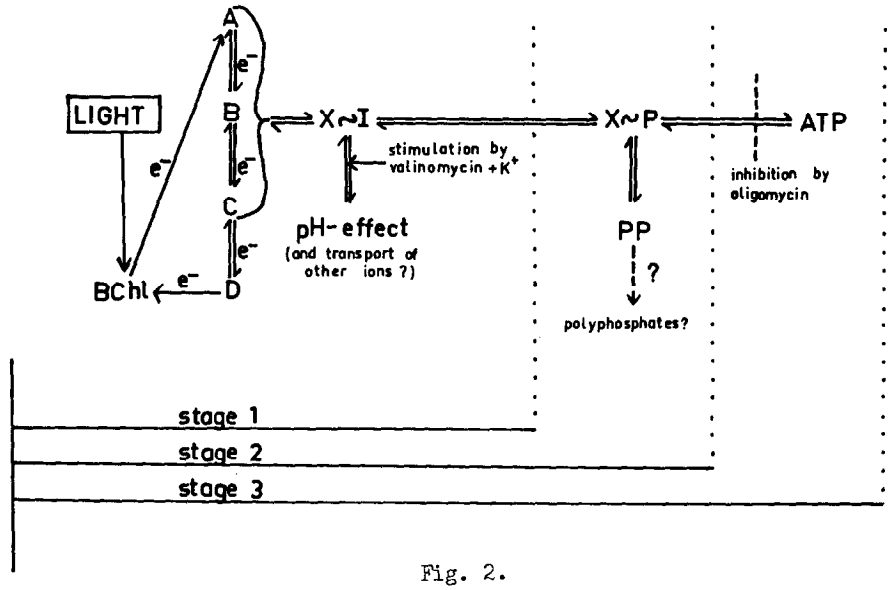

Fig. 2.

simplicity depicts energy transfer from only one of the two existing coupling sites, i.e. that from which valinomycin-sensitive energy transfer reactions emerge (Baltscheffsky and von Stedingk, 1966). The scheme in Fig. 2, in which also the suggested pathway of the light-induced pH-change is incorporated (von Stedingk and Baltscheffsky, 1966), shows the hypothetical phosphorylated intermediate X~P and pyrophosphate in equilibrium. The slow kinetics of its formation as compared with that of ATP-formation, indicates that it is not identical with X~P but on a side-path emerging from this compound. The data obtained with oligomycin would appear to eliminate the possibility that light-induced formation of pyrophosphate results from ATP formed from endogenous ADP, of which only very minute amounts exist in isolated chromatophores. Support for the concept of a true equilibrium between pyrophosphate and X~P comes from the demonstrated rapid decay of phosphorylated product in the dark (Horio, von Stedingk and Baltscheffsky, 1966) and the finding that certain uncoupling agents stimulate the structure-bound pyrophosphatase reaction of the chromatophores (M. Baltscheffsky, 1964; M. Baltscheffsky and H. Baltscheffsky, in preparation).

Besides reviving early claims about respiration-linked pyrophosphate-formation (Cori, 1942; Cross, Taggart, Covo and Green, 1949; Lindberg and Ernster, 1952) as well as about its origin (Kornberg, 1950) the demonstration of light induced formation of pyrophosphate at an earlier stage of energy transfer than the formation of ATP brings into focus the demonstration, more than a decade ago, of light-induced formation of polyphosphate in Chlorella (Wintermans, 1955) and the recent observations by Hughes and his collaborators (Hughes, Conti and Fuller, 1963; Cole and Hughes, 1965) that photophosphorylating extracts from Chlorobium thiosulfatophilum contain an enzyme which reversibly transfers phosphate groups from polyphosphate to ADP, giving ATP. However, no formation of polyphosphate has been observed in the present studies and thus the suggested pathway to polyphosphate in Fig. 2 is only a speculation. The demonstration that pyrophosphate may serve as phosphate donor to yield

energy-rich phosphate (Siu and Wood, 1962) supports the concept of a rapid equilibrium between X~P and pyrophosphate.

The main result appearing to emerge from the data presented and their interpretation is that the photosynthetic system of bacterial chromatophores now allows experimental analysis of three consecutive stages of electron transport-coupled energy transfer leading to ATP (Fig. 2). This property of the chromatophores would seem at present to be unique among the known subcellular electron transport phosphorylation systems of photophosphorylation and oxidative phosphorylation,

ACKNOWLEDGEMENTS. This work has been supported by grants from the Charles F. Kettering Foundation, Dayton, Ohio, U.S.A. and The Swedish Natural Science Research Council. We thank Professor Olov Lindberg for laboratory space and Miss Ulla Lundgren for skilful assistance.

REFERENCES

Avron, M., Biochim.Biophys.Acta 40, 257 (1960).
Baltscheffsky, H., Biochim.Biophys.Acta 40, 1 (1960).
Baltscheffsky, H. and Baltscheffsky, M., Acta Chem.Scand. 14, 257 (1960).
Baltscheffsky, H. and Arwidsson, B., Biochim.Biophys.Acta 65, 425 (1962).
Baltscheffsky, H. and von Stedingk, L.-V. in Currents in Photosynthesis, Eds. J.B. Thomas and J.C. Goedheer, Ad. Donker, Rotterdam, (in press) (1966).
Baltscheffsky, H., von Stedingk, L.-V., Heldt, H.W. and Klingenberg, M. (in preparation).
Baltscheffsky, M. in Federation of European Biochemical Societies 1st Meeting, London, Abstracts, p. 67 (1964).
Baltscheffsky, M. and Baltscheffsky, H. (in preparation).
Cole, J.A. and Hughes, D.E., J.Gen.Microbiol. 38, 65 (1965).
Cori, C.F. in A Symposium on Respiratory Enzymes, Madison, p. 175 (1942).
Cross, R.J., Taggart, J.V., Covo, G.A. and Green, D.E., J.Biol.Chem. 177, 655 (1949).
Grande, J.A. and Beukenkamp, J., Anal.Chem. 28, 1497 (1956).
Horio, T., von Stedingk, L.-V. and Baltscheffsky, H., Acta Chem,Scand. 20, 1 (1966).
Hughes, D.E., Conti, S.F. and Fuller, R.C., J.Bacteriol. 85, 577 (1963).
Kornberg, A., J.Biol.Chem. 182, 779 (1950).
Lindberg, O. and Ernster, L., Exptl.Cell Res, 3, 209 (1952).
Lindenbaum, S., Peters, V. and Rieman, W., Anal.Chim.Acta 11, 530 (1954).
Siu, P.M.L. and Wood, H.G., J.Biol.Chem. 237, 3044 (1962).
von Stedingk, L.-V. (in preparation).
von Stedingk, L.-V. and Baltscheffsky, H., (in preparation).
Wintermans, J.F.G.M. Mededel.Landbouwhogeschool Wageningen 55, 69 (1955).