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REGULATION OF CYCLIC PHOTOPHOSPHORYLATION IN *RHODOSPIRILLUM RUBRUM* BY THE REDOX STATE OF NICOTINAMIDE-ADENINE DINUCLEOTIDE

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Summary

We have investigated the effect of the redox state of added NAD on the rates of anaerobic cyclic photophosphorylation which are supported by membrane vesicles isolated from *Rhodospirillum rubrum*. As the redox potential of NAD was lowered, the activity decreased according to a typical potentiometric titration. The Nernst plot showed an apparent midpoint potential (E'_0) of —350 mV and had a slope which corresponded to a two-electron transition. Besides, an almost identical potentiometric relationship was found to exist between the extent of light-elicited ATP formation in anaerobic suspensions of intact *R. rubrum* cells and the redox potential of intracellular NAD. These results suggest that physiological photophosphorylation in *R. rubrum* requires the oxidized form of a membrane-bound constituent (E'_0 = —350 mV) whose redox state is controlled by the redox state of cytoplasmic NAD.

Introduction

Some years ago, it was proposed that bacterial cyclic photophosphorylation might be physiologically regulated by changes in the cytoplasmic levels of redox metabolites [1]. The proposal was based on the observation that the photophosphorylation activity of membrane vesicles (chromatophores) isolated from photosynthetic bacteria depended on the redox potential of the assay mixture [1—4]. Recently we have shown that anaerobic photophosphorylation in intact *Rhodospirillum rubrum* cells is inhibited under conditions which favour the intracellular accumulation of metabolic reductants, and that low levels of oxygen relieve the inhibition [5], thus confirming the physiological

significance of the previous in vitro observations. Our experiments have also shown that the extent of anaerobic photophosphorylation in intact cells is proportional, at least within a certain range, to the fraction of intracellular NAD which is in the oxidized state [6]. In the present report it is demonstrated that a similar relationship exists between the rates of photophosphorylation which are supported by isolated *R. rubrum* chromatophores and the redox state of added NAD. These results indicate that the effect of the nucleotide on phosphorylative activity is direct and suggest, in addition, that the redox state of internal NAD plays an important role in the physiological regulation of cyclic photophosphorylation in *R. rubrum*.

Materials and Methods

Rhodospirillum rubrum, strain S1, was cultured photoanaerobically as previously described [7]. The growth medium was that of Lascelles [8] supplemented with 2 g/l yeast extract. Cells were collected at the exponential phase of growth when the cultures reached cell densities of 1.8 mg dry weight/ml and specific bacteriochlorophyll contents of about 3—3.5 nmol/mg dry weight.

Photosynthetic membrane vesicles (chromatophores) were prepared according to a previously published procedure [7] with two modifications: (i) cell batches of 3 g wet weight were ground with 7.5 g alumina powder for 1 min in a mortar equipped with a motor-driven pestle (RMO, Retsch); and (ii) final chromatophore preparations were suspended in a mixture of 50 mM Tricine-NaOH (pH 8.0) and glycerol (1 : 1, v/v) and stored at −35°C in the dark. No detectable loss of phosphorylative activity was observed for two months.

The reaction mixture for the assay of anaerobic cyclic photophosphorylation has been described already [6]. It contained chomatophores, buffer, photophosphorylation substrates and an oxygen-scavenger system. NAD^+ was also present at variable concentrations. The mixtures were covered with argon-gassed paraffin oil and left in the dark for 20 min at 25°C to ensure removal of all traces of O_2 and H_2O_2. Then the required amount of NADH was added in a small volume of an argon-gassed solution (total NAD concentration, 1 mM) and dark incubation was continued for 30 min. (It was previously checked that after 20 min in the presence of NADH, phosphorylative activity remained constant for at least 15 min more). Then the mixtures were illuminated with a slide projector equipped with a 150 W tungsten-halide lamp and a broadband infrared filter (IR-total, Balzers). After additional filtering through 10 cm water, the light intensity reaching the assay mixtures was 43 $W \cdot m^{-2}$. At the end of illumination (3 min), each reaction mixture received $HClO_4$ (1 M final concentration), was kept in ice for 10 min and neutralized with KOH to pH 7.4. After filtration to remove solid debris, the ATP content of the solution was estimated with the aid of hexokinase (EC 2.7.1.2), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and $NADP^+$ [7]. The amount of NAD^+ present was also analyzed enzymatically [6] in a separate aliquot and found always to correspond, within experimental error, to that initially added.

During the prolonged incubation, required to ensure anaerobiosis and redox equilibration, a substantial amount of ATP was formed even in the absence of inorganic phosphate, probably from ADP by the adenylate kinase activity of

chromatophores. The extent of this ATP formation ranged from 40 to 55 mol per mol bacteriochlorophyll and was independent of the ratio of NAD^+ to NADH. For each particular set of assays, it was estimated in a separate reaction mixture without phosphate and substracted from the ATP content of the complete mixtures.

Results and Discussion

NADH is an efficient inhibitor of the photophosphorylative activity of isolated *Rhodospirillum rubrum* chromatophores under strictly anaerobic conditions [9]. When the activity was assayed in the presence of mixtures of NAD^+ and NADH, the extent of the inhibition depended upon the ratio of NADH to total NAD (Fig. 1) and not upon the concentration of NADH alone, which was always kept above the lowest one required to elicit maximal inhibition in the absence of NAD^+ [6]. A semilogarithmic representation of relative activity versus the potential of the NAD^+-NADH mixture yields a linear plot (Fig. 2), which is consistent with a typical potentiometric titration. As depicted in Fig. 2, the apparent midpoint redox potential is about −350 mV (pH 8.0) and the magnitude of the slope of the plot corresponds closely to a two-electron transition. In order to evaluate the physiological significance of these results, it seemed interesting to check whether there existed in vivo a similar dependence of photophosphorylation on the redox state of intracellular NAD. With that aim, we reexamined previous experiments, carried out with suspensions of intact cells, in which both the extent of light-elicited ATP formation and the redox state of internal NAD had been determined [6]. The use of those

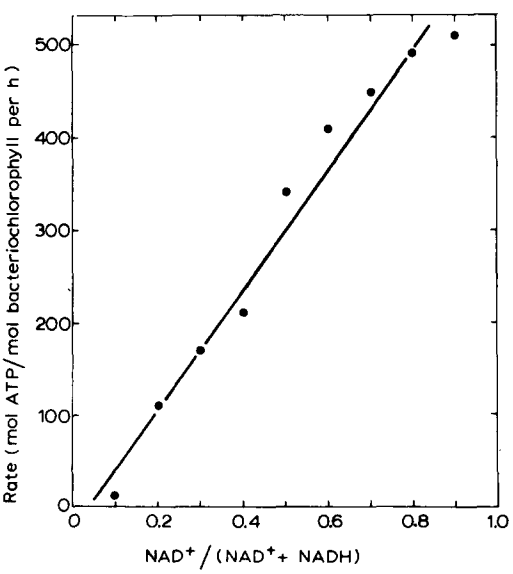

Fig. 1. Effect of the redox state of NAD on the phosphorylative activity of *R. rubrum* chromatophores. Assays were carried out as described under Methods. The least-square method was used to adjust the experimental points to the straight line (correlation coefficient, 0.984).

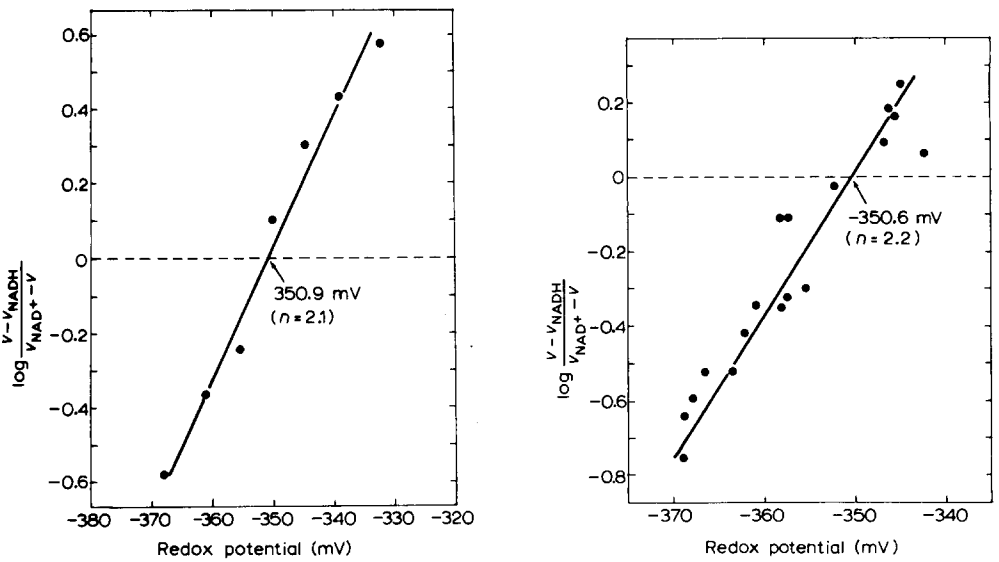

Fig. 2. Potentiometric titration of chromatophore photophosphorylation using NAD as a redox buffer. Experimental data are the same as in Fig. 1. Maximal (v_{NAD^+}) and minimal (v_{NADH}) activities were obtained by extrapolation of the linear plot in Fig. 1. The least-square method was used to draw the straight line (correlation coefficient, 0.987).

Fig. 3. Dependence of light-elicited ATP formation in intact cells on the redox potential of intracellular NAD. The experimental data and values of maximal (v_{NAD^+}) and minimal (v_{NADH}) ATP formation were taken from Fig. 3 in Ref. 6. Anaerobic cell suspensions in growth medium were kept in the dark for 30 min. The ATP content of those suspensions after 30 s of illumination was taken as an approximate estimation of the rate of ATP formation in vivo. The redox potential of intracellular NAD was estimated from the levels of NAD^+ and NADH, which were extracted before illumination from duplicate cell suspensions. The least-square method was used to adjust the experimental points to the straight line (correlation coefficient, 0.958).

data to build a potentiometric plot comparable with that of Fig. 2 required two assumptions: (i), the rate of photophosphorylation in vivo may be estimated to a good approximation from the ATP levels reached by anaerobic cell suspensions after brief illumination (cf. Ref. 5); and (ii) the redox state of the fraction of intracellular NAD which may interact with the photochemical system is close to the redox state of total NAD. On the basis of both assumptions, the plot shown in Fig. 3 was built. Its parameters (slope and midpoint potential) resulted almost identical to those of the plot in Fig. 2. Since Fig. 2 was drawn using actual photophosphorylation rates and prefixed NAD redox potentials, that similarity supports strongly the validity of the assumptions made to build the plot of Fig. 3. Therefore, it seems reasonable to conclude that there exists a simple potentiometric relationship between the photophosphorylative activity of intact *R. rubrum* cells and the redox state of intracellular NAD.

Among the explanations which may be advanced to account for the effect of the redox state of NAD on *R. rubrum* photophosphorylation, perhaps the simplest one is to assume that, prior to illumination, the nucleotide reaches redox equilibration with an endogenous constitutent which is in a 1 : 1 ratio to the photosynthetic unit, and that only those photosynthetic units in which such a constituent is oxidized are active upon subsequent illumination. The

linearity of the plots in Figs. 2 and 3 and the magnitude of their slopes are consistent with the involvement of some type of redox equilibrium. On the other side, it should be remarked here that those potentiometric plots correspond, within experimental error, to that expected from the titration of NAD itself (E'_o = −350 mV at pH 8.0; n = 2). The resemblance seems too high to be merely coincidental and suggests that the nucleotide, perhaps as the cofactor of a membrane-bound component, is the electron carrier which, as proposed above, is required in its oxidized form for the photosynthetic system to be functional. In this respect it appears important to investigate the possible involvement of NAD in the primary and secondary photochemical reactions which lead to physiological photophosphorylation in *R. rubrum*.

The range of redox potentials in which photophosphorylation is attenuated by NAD (Figs. 2 and 3) falls within the range which allows primary bacterial photochemistry [10]. However, it is located below −100 mV, the lower limit of the potential range within which, according to the previous titrations [4,11], isolated *R. rubrum* and *Rhodopseudomonas sphaeroides* chromatophores are abie to photophosphorylate. We think that the discrepancy is mainly due to the different properties of the redox buffers used in each case to adjust the potential of the system prior to the assay of its activity. If it is realized that different added buffers may interact specifically with different endogenous constituents of the photosynthetic apparatus, the dependence of the titration on the nature of the buffer may be understood. Another alternative possibility, namely that the discrepancy in the results arises from differences in the chromatophore preparations, does not seem likely because artificial redox mediators, used as buffers in the previous titrations [4,11], inhibit similarly photophosphorylation in our chromatophore preparations within the potential range which is buffered by mixtures of NAD^+ and NADH (Table I).

The mechanism proposed here to account for the effect of the redox state of NAD on photophosphorylative activity does not necessarily imply that the hypothetical constituent interacting with NAD participates directly in cyclic electron transfer. Such a constituent may be instead a regulating factor which controls, through changes in its redox state, the rate of some intermediate step (redox or other) of cyclic photophosphorylation. Several groups [12—14] have proposed the existence in mitochondria of such a type of redox modulator which controls the reduction of cytochromes *b*. However, it is also possible that NAD influences the rate of photophosphorylation through its direct inter-

TABLE I

EFFECT OF SOME REDOX CARRIERS ON THE RATE OF PHOTOPHOSPHORYLATION ASSAYED IN THE PRESENCE OF PARTLY REDUCED NAD *

Redox carrier	Phosphorylation rate (mol ATP · h^{-1} · mol^{-1} bacteriochlorophyll)
None	405
50 μM FMN	50
50 μM diaminodurene	135
75 μM 2,6-dichlorophenolindophenol	67

* $NAD^+/(NAD^+ + NADH) = 0.7$

action with a carrier located in the main pathway of cyclic electron transport. If that were so, the endogenous carrier might be either the primary electron acceptor or a secondary acceptor involved in a subsequent redox step. Since the reported redox properties of the primary acceptor [10] do not agree with those of the constituent titrable with NAD (Figs. 2 and 3), we favour the second interpretation. The previous reduction of a secondary acceptor by the external buffer would prevent the normal reoxidation of the photoreduced primary acceptor, which would either remain trapped in its reduced form or transfer an electron to the photooxidized primary donor in a useless back reaction, depending on whether reduction of the primary donor had already taken place or not. In either case, cyclic electron flow would not be possible. It should be remarked here that inhibition of sustained bacterial photochemistry by the exogenous reduction of secondary electron acceptors has been reported by other authors [15].

A different aspect which deserves also some comments is the possible physiological meaning of the regulation of photophosphorylation by the redox state of NAD. *R. rubrum*, like other *Rhodospirillaceae*, is a photoheterotroph whose net requirements for metabolic reductants and oxidants are not high and depend mainly on the redox level of carbon in the organic growth substrate as compared to the redox level of carbon in cell material. Therefore, and in contrast to plant photosynthesis, *R. rubrum* photosynthesis is essentially devoted to ATP production through the cyclic electron transport system while most of the redox metabolites may be directly obtained from the carbon source [16]. Then, the organism should coordinate in some way photosynthesis and dark metabolism so that ATP and redox metabolites are produced at the rates which are optimal for growth under the actual culture conditions. In view of the experimental data described here, it seems that NAD, a redox carrier which participates in many reactions of intermediary metabolism, may play a key role in some aspects, at least, of such a coordination. However, full understanding of the physiological meaning of the regulation of phosphorylative activity by the redox state of NAD would require detailed knowledge of how the redox state of the nucleotides may change under the variety of culture conditions possible for *R. rubrum*.

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References

1 Bose, S.K. and Gest, H. (1963) Proc. Natl. Acad. Sci. U.S. 49, 337–345
2 Newton, J.W. and Kamen, M.D. (1957) Biochim. Biophys. Acta 25, 462–474
3 Geller, D.M. and Lipmann, F. (1960) J. Biol. Chem. 235, 2478–2484
4 Culbert-Runquist, J.A., Hadsell, R.M. and Loach, P.A. (1973) Biochemistry 12, 3508–3514
5 Giménez-Gallego, G., del Valle-Tascón, S. and Ramírez, J.M. (1976) Arch. Microbiol. 109, 119–125
6 Giménez-Gallego, G., del Valle-Tascón, S. and Ramírez, J.M. (1978) Z. Pflanzenphysiol. 87, 25–36

7 del Valle-Tascón, S. and Ramírez, J.M. (1975) Z. Naturforsch. 30c, 46—52
8 Lascelles, J. (1956) Biochem. J. 62, 78—93
9 Vernon, L.P. and Ash, O.K. (1960) J. Biol. Chem. 235, 2721—2727
10 Loach, P.A. (1976) in Progress in Biorganic Chemistry (Kaiser, E.T. and Kezdy, F., eds.), vol. 4, pp. 89—192, Wiley, New York, NY
11 Loach, P.A., Sponholtz, D.K. and Querfurth, H. (1977) Fed. Proc. 36, 880
12 Rieske, J.S. (1971) Arch. Biochem. Biophys. 145, 179—193
13 Eisenbach, M. and Gutman, M. (1975) Eur. J. Biochem. 52, 107—116
14 Trumpower, B.L. and Katki, A. (1975) Biochem. Biophys. Res. Commun. 65, 16—23
15 Loach, P.A., Kung, M.C. and Hales, B.J. (1975) Ann. N.Y. Acad. Sci. 244, 297—319
16 Stanier, R.Y. (1961) Bacteriol. Rev. 25, 1—17