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THE INFLUENCE OF ENERGY-TRANSFER INHIBITORS ON PROTON PERMEABILITY AND PHOTOPHOSPHORYLATION IN NORMAL AND PREILLUMINATED *RHODOSPIRILLUM RUBRUM* CHROMATOPHORES

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Summary

(1) Chromatophores were preilluminated in the presence of phenazine methosulphate or diaminodurene, and without phosphorylation substrates; next they were transferred to fresh medium and assayed for light-induced proton uptake, light-induced 9-aminoacridin fluorescence quenching, and photophosphorylation.

(2) Preillumination in the presence of phenazine methosulphate or diaminodurene causes an inhibition of the photophosphorylation rate. The presence of ADP + $MgCl_2$ + phosphate, or ADP + $MgCl_2$ + arsenate during preillumination provides full protection against this effect.

(3) Preilluminated chromatophores are leaky for protons. The leak is expressed as an accelerated dark decay, and a diminished extent of succinate-supported, light-induced proton uptake. The extent of light-induced 9-aminoacridin fluorescence quenching is also diminished.

(4) The proton leak can be closed by oligomycin and by dicyclohexyl carbodiimide (at concentrations similar to those used to inhibit photophosphorylation), but not by aurovertin. Closure of the proton leak results in partial restoration of the photophosphorylation rate.

(5) The inhibition of phosphorylation by oligomycin or dicyclohexyl carbodiimide is time-dependent. In untreated chromatophores, the time-dependence is determined by the extent of membrane energization. In preilluminated chromatophores, the time-dependence is determined in addition by the extent

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Abbreviations: DCCD, *N,N'*-dicyclohexyl carbodiimide; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; Bchl, bacteriochlorophyll.

to which the proton leaks have been closed. The reasons for this are briefly discussed.

Introduction

In a previous publication [1], we reported on a light-induced inhibition of photophosphorylation and dark ATPase activity in *Rhodospirillum rubrum* chromatophores. The inhibition was induced by preillumination of chromatophores with far-red light in the presence of phenazine methosulphate, and under non-phosphorylation conditions. This observation was confirmed recently [2]. Complete protection against photo-inactivation was provided by the simultaneous presence of ADP, P_i and $MgCl_2$ during preillumination [1], and over 85% protection was provided by uncouplers, such as CCCP [1], or valinomycin plus nigericin in the presence of K^+. (valinomycin plus K^+ or nigericin plus K^+ offered only 10—20% protection against photo-inactivation) (Slooten, L., unpublished results).

These, and other results indicated that phenazine methosulphate-mediated photo-inactivation occurred whenever a lack of substrate(s) precluded photophosphorylation, while the membrane remained highly energized. It was suggested that the reaction(s) leading to photoinactivation might be a 'side-path' of the reactions leading to photophosphorylation.

We felt that we might gain some insight into various aspects of the coupling mechanism of phosphorylation from: (a) an investigation of the conditions under which the photoinactivation occurs or is prevented, and (b) an investigation of the properties of photoinactivated chromatophores, as compared to normal chromatophores. This paper deals with some results obtained with these lines of attack.

Methods

Preparation of chromatophores

R. rubrum, strain S1, was grown on the medium described by Cohen-Bazire et al. [3], except that succinate was also present (at 15 mM), and casamino acids (casein hydrolysate) were replaced by yeast extract (Difco, 2 g/l) and peptone (Difco, 5 g/l). The cells were collected by centrifugation after 3 days of growth in 1.2-l Roux bottles, in weak incandescent light. The cells were washed twice by resuspension and centrifugation) in a medium containing 50 mM KCl and 10 mM Tris-HCl, pH 7.5. The washed cells were broken by sonication as described before [1], in a medium containing 0.3 M sucrose and 0.1 M glycylglycine-KOH, pH 7.5. The suspension was centrifuged and the fraction sedimenting between 20 000 × *g* (30 min and 100 000 × *g* (60 min) was collected and washed twice in a medium containing 0.1 M sucrose, 25 mM KCl, 0.1 mM EDTA and 10 mM glycylglycine-KOH, pH 8.0 (EDTA was omitted during the second washing). The washed chromatophores were stored at 0° C in a medium containing 90 mM KCl and 20 mM glycylglycine-KOH (pH 8.0) at a bacteriochlorophyll concentration of approx. 1 mM. These preparations will be called hereafter 'untreated chromatophores'.

Preillumination of chromatophores

Preillumination was carried out in the reaction vessel for pH measurements [1], under aerobic conditions and (unless otherwise indicated) in a medium containing, in a final volume of 5.7 ml, 0.2 M sucrose, 30 mM KCl, 0.1 mM EDTA, 3 mM glycylglycine-NaOH, 0.4 mM succinate, 0.4 mM phenazine methosulphate and chromatophores corresponding to 14—26 μM bacteriochlorophyll. The final pH was 7.3. The preillumination time was 3 min. The light was provided by a 100 W tungsten-iodine lamp and was filtered through 10 cm water and through an orange cut-off filter (Far-red light could be used only in dilute chromatophore suspensions [1]). Control chromatophores were suspended in the same medium but were kept in the dark. The preilluminated and control chromatophores were sedimented by centrifugation. The supernatant was discarded, the tube walls were rinsed and the chromatophores resuspended and stored for 0—5 days at 0°C, in a minimal amount of a medium containing 0.2 M sucrose, 30 mM KCl, 0.1 mM EDTA and 10 mM glycylglycine-NaOH, final pH 7.8.

Measurements

Photophosphorylation and light-induced proton uptake were measured aerobically with the pH method [4] as described [1]. The light was provided by a 100 W tungsten-iodine lamp and was filtered through 10 cm water containing 15 mg/ml $NiSO_4$, and through a far-red cut-off filter. The light intensity was 45 mW. 9-Aminoacridin fluorescence was measured in a rectangular, stirred cuvette with an optical path of 11.5 mm. The wavelength of the (weak and modulated) excitation light was approx. 402 nm. The fluorescence emitted at right angles to the excitation light was focussed onto a Philips XP-1004 photomultiplier, after passing through a filter combination which transmitted wavelengths between approx. 460 and 700 nm. Continuous, actinic light from a 100 W tungsten-iodine lamp was filtered through 2 cm water containing 75 mg/ml $NiSO_4$ and through a far-red cut-off filter. The light intensity in the cuvette was 70 mW. The zero percent quenching level was determined after addition of 0.04% Triton X-100 [5]. Bacteriochlorophyll was estimated spectrophotometrically, using an in vivo extinction coefficient of 140 $mM^{-1} \cdot cm^{-1}$ at 880 nm [6]. Light intensities were measured with the ferrioxalate actinometer procedure [7], and with a calibrated thermopile.

Materials

Valinomycin, oligomycin, ADP and ATP were from Sigma. Aurovertin was a gift from Prof. E.C. Slater, University of Amsterdam. Diaminodurene was a gift from Prof. A. Trebst, Ruhr-University, Bochum, F.R.G. Nigericin was a gift from the Eli Lilly Laboratories, Indianapolis, IN. All other reagents were analytical grade. The antibiotics were added as ethanol solutions. The ethanol concentration was kept below 5 ‰ (v/v) in each experiment.

Results

We reported previously [1] that the simultaneous presence of ADP, P_i and $MgCl_2$ during preillumination completely prevented photoinactivation. Table I

TABLE I

PROTECTIVE EFFECT OF ARSENATE AGAINST PHOTOINACTIVATION

Chromatophores were preilluminated as described in Methods, except that in the dark control and during preillumination, ADP and $MgCl_2$ were present at concentrations of 0.5 and 3 mM, respectively; phosphate (3 mM) and arsenate (3 mM) were present during preillumination when indicated in the table. After preillumination, the concentrations of both phosphate and arsenate were adjusted to 3 mM in all samples (including the dark control). After centrifugation, photophosphorylation was assayed in a medium with 0.2 M sucrose, 30 mM KCl, 0.1 mM EDTA, 3 mM $MgCl_2$, 3 mM sodium phosphate, 0.5 mM ADP, 0.4 mM succinate and (if present) 0.4 mM phenazine methosulphate. The final pH was 7.3. The bacteriochlorophyll concentration during the assay was approx. 4 μM.

Pretreatment	Photophosphorylation rate ($\mu M\ P_i \cdot mg^{-1}$ Bchl $\cdot min^{-1}$)	
	+ succinate	+ succinate + phenazine methosulphate
Dark control + ADP + $MgCl_2$	1.25	5.44
Preilluminated + ADP + $MgCl_2$	0.64	2.61
Preilluminated + ADP + $MgCl_2$ + As	1.12	5.44
Preilluminated + ADP + $MgCl_2$ + P_i	1.22	5.58

shows that during preillumination in the presence of ADP and $MgCl_2$, arsenate is approximately as effective as phosphate in providing protection against photoinactivation. Subsequent experiments revealed that arsenate provided no significant protection against photoinactivation, when either $MgCl_2$ or ADP, or both, were omitted during preillumination. In this respect, too, arsenate behaves like phosphate (not shown). Thus, only phosphorylation conditions and 'arsenylation' conditions offer complete protection against phenazine methosulphate-mediated photo-inactivation. This supports earlier suggestions [5,8] that arsenate is a phosphate analogue with respect to its interactions with the chloroplast and bacterial coupling factor.

Photo-inactivation of phosphorylation was also observed, using diaminodurene, instead of phenazine methosulphate, during preillumination. After preillumination in the presence of diaminodurene, two effects could be discerned: (1) chromatophores preilluminated under phosphorylation conditions were slightly inactivated with respect to the dark controls (Table II, lines 3 and 4). This effect was specific for diaminodurene, since it was not observed after phenazine methosulphate-preillumination (Table I). (2) Omission of ADP during diaminodurene-preillumination resulted in a more extensive inhibition of phosphorylation, as appears from the 4th and 5th lines of Table II. This second type of inhibition was about equally extensive as the inhibition caused by phenazine methosulphate-preillumination under non-phosphorylation conditions (Table II, lines 1 and 2). Like the phenazine methosulphate-mediated photo-inactivation [1], the second type of diaminodurene-mediated photoactivation was completely prevented by addition of 15 μM CCCP prior to preillumination (not shown).

The relatively low extents of photo-inactivation reported in Tables I and II (40—50%) were due to the presence of $MgCl_2$ during preillumination. This provided a slight protection against photo-inactivation (not shown). After preillumination as described in Methods, photophosphorylation (assayed as described in Table I) was 64—77% inhibited in 16 preparations. The succinate-supported phosphorylation rates in the control chromatophores of these prep-

TABLE II

COMPARISON OF DIAMINODURENE-MEDIATED WITH PHENAZINE METHOSULFATE-MEDIATED PHOTOINACTIVATION OF PHOTOPHOSPHORYLATION

Preillumination was carried out as described in Methods, except that, where indicated, succinate + phenazine methosulfate were replaced by 1.05 mM diaminodurene. In the dark controls and during preillumination, sodium phosphate and $MgCl_2$ were present at 3 mM each. ADP (0.5 mM) was present during preillumination as indicated in the table. After preillumination ADP was added to give a final concentration of 0.5 mM in all samples (including the dark controls). After centrifugation, photophosphorylation was assayed as described in the legend to Table I. All experiments were done with the same batch of chromatophores.

Pretreatment	Photophosphorylation rate			
	$\mu M\ P_i \cdot mg^{-1}$ Bchl $\cdot min^{-1}$		Inhibition (%)	
	+ succinate	+ succinate + phenazine methosulfate	+ succinate	+ succinate + phenazine methosulfate
Dark control + succinate + phenazine methosulfate	4.08	10.5	0	0
Preilluminated + succinate + phenazine methosulfate	2.47	5.85	39 *	44 *
Dark control + diaminodurene	4.02	9.02	0	0
Preilluminated + diaminodurene + ADP	3.15	8.25	22 *	9 *
Preilluminated + diaminodurene	1.64	4.50	48 **	45 **

* With respect to the dark control

** With respect to the sample preilluminated under phosphorylation conditions (4th line).

arations varied between 0.26 and 4.2 $\mu M\ P_i \cdot mg^{-1}$ Bchl $\cdot min^{-1}$ (the lowest values were observed in controls prepared from aged, untreated chromatophores). There was no correlation between these phosphorylation rates, and the extent of phenazine methosulphate-mediated photo-inactivation. This makes sense, because the variations in phosphorylation rates of at least fresh chromatophores were mainly due to extensive, but irreproducible losses of cytochromes during the breaking of the cells [9]. However, the cytochrome region of the electron transport chain is not involved in the reactions leading to photo-inactivation, since saturating concentrations of antimycin A provided no protection against phenazine methosulphate-mediated photo-inactivation of succinate plus phenazine methosulphate-supported photophosphorylation (not shown).

We reported previously [1], that light-induced proton uptake was not strongly inhibited in chromatophores preilluminated with phenazine methosulphate, as compared to dark control chromatophores. However, in those experiments, light-induced proton uptake was measured in the presence of phenazine methosulphate and under non-phosphorylation conditions. Conse-

quently, during assays at pH 8.0, the control chromatophores where photoinactivated during the measurement of light-induced proton uptake. Therefore we have repeated these experiments, using different assay conditions. The present measurements were done in the presence of succinate. Moreover, valinomycin was usually added at 0.1 μM, because in its presence the light-induced electrochemical proton gradient across the membrane consists predominantly of a ΔpH [10,11]. This renders ΔpH indicators (see below) more useful as indicators of the light-induced electrochemical proton gradient across the membrane.

Fig. 1 shows some kinetics of succinate-supported, light-induced proton uptake in preilluminated and control chromatophores. In the absence of oligomycin, preilluminated chromatophores exhibited an accelerated dark decay, and a reduced extent of the light-induced proton uptake, as compared to control chromatophores. Addition of oligomycin caused a decrease in the dark decay rate, and an increase in the extent of light-induced proton uptake in preilluminated chromatophores. This indicates that preilluminated chromatophores are leaky for protons. The leak can be blocked with oligomycin. Similar results, but with 40—60% lower extents of light-induced proton uptake, and with somewhat less marked differences between preilluminated and control chromatophores in the absence of oligomycin, were obtained when valinomycin was omitted from the assay mixture (not shown). These results were not further analyzed, because measurements of succinate-supported, light-induced proton uptake in the absence of valinomycin caused a slight, irreversible inhibition of photophosphorylation (10—15%) in control chromatophores [1]. Valinomycin at 0.1 μM provided a complete protection against this effect (not shown).

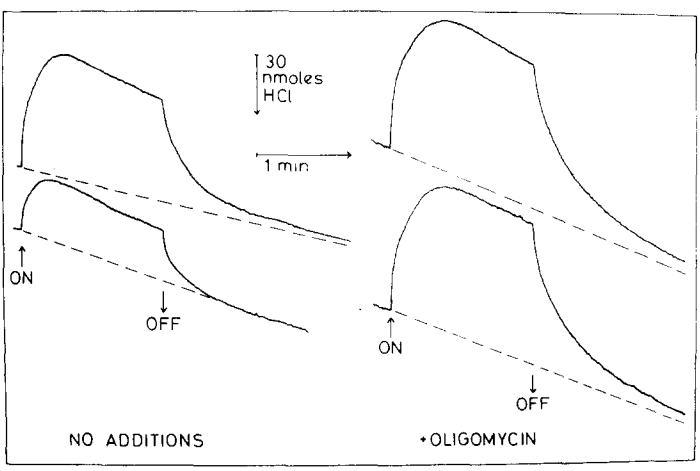


Fig. 1. Kinetics of light-induced pH changes in suspensions of preilluminated chromatophores (bottom) and control chromatophores (top), without oligomycin (left), and in the presence of 1.8 μg/ml oligomycin (right). The assay medium contained 0.2 M sucrose, 30 mM KCl, 0.1 mM EDTA, 2 mM glycylglycin, 0.8 mM sodium succinate, 0.1 μM valinomycin, 7.5 μg 9-aminoacridin, chromatophores corresponding to approx. 14 μM Bchl, and NaOH to give a final pH of 7.8. The volume was 5 ml. Chromatophores were incubated in the complete reaction medium for 12 min prior to the switching on of the light. ------, base line, obtained by extrapolation of the pH change observed prior to the switching on of the light.

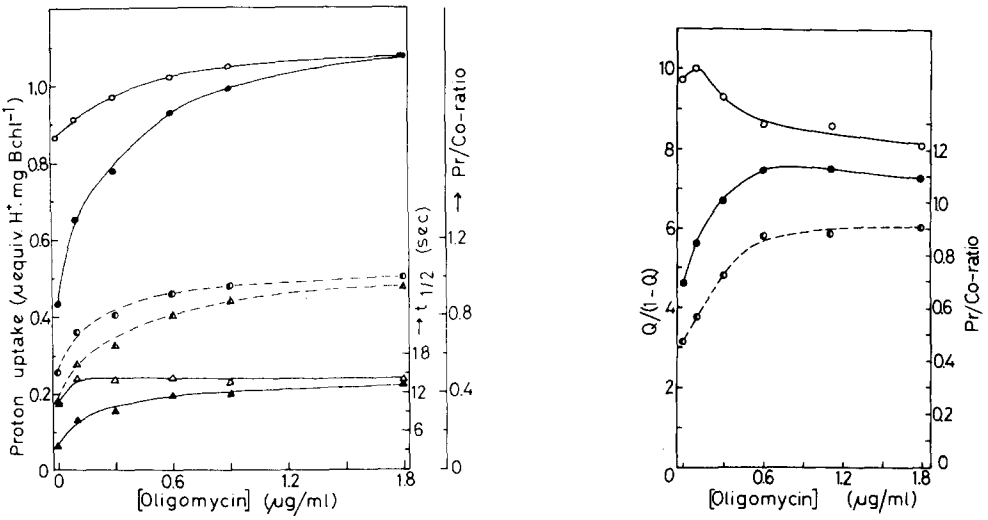


Fig. 2. Oligomycin-dependence of succinate-supported, light-induced proton uptake in preilluminated (●, ▲) and control (○, △) chromatophores, ○———○ and ●———●, maximum extent of the light-induced proton uptake. △———△ and ▲———▲, half time of the dark decay of light-induced proton uptake. ●· · · · ·●, Pr/Co ratio of the extents of light-induced proton uptake (definition, see text). ▲· · · · ·▲, Pr/Co ratio of the half-times of the dark decay of light-induced proton uptake. The assay was carried out as described in the legend to Fig. 1.

Fig. 3. Oligomycin-dependence of the steady-state extent of the quenching ratio (Q/1 — Q) of light-induced 9-aminoacridin fluorescence quenching in preilluminated (●) and control (○) chromatophores. The values of Q/1 — Q were corrected for slight deviations of the Bchl concentrations from 14 μM, by means of a calibration curve in which (Q/1 — Q) was plotted against the bacteriochlorophyll concentration. ●· · · · ·●, Pr/Co ratio of the steady-state extents of (Q/1 — Q). The assay was carried out as described in the legend to Fig. 1, except that glycylglycine was present at 10 mM, and 9-aminoacridin was added at 2 min prior to the switching on of the actinic light.

Fig. 2 shows the oligomycin-dependence of light-induced proton uptake in preilluminated and control chromatophores. In order to illustrate the results more clearly, Fig. 2 also shows the ratio of the extents of light-induced proton uptake in preilluminated and control chromatophores (hereafter denoted: the Pr/Co ratio of the extents of light-induced proton uptake). This ratio increased from 0.50 (in the absence of oligomycin) to 1.00 (at saturating concentrations of oligomycin). The Pr/Co ratio of the half-times of the dark decay of light-induced uptake increased from 0.35 (without oligomycin) to 0.95 (at saturating concentrations of oligomycin). The curves of the Pr/Co ratios indicate that the effect of oligomycin on the closure of the proton leak in preilluminated chromatophores was half-maximal at a concentration of about 0.2 μg/ml, and was almost saturated at 0.9 μg/ml.

Aurovertin, at 0.9 μM, did not significantly influence the kinetics or the extent of light-induced proton uptake in either preilluminated or control chromatophores. The concentration of 0.9 μM was saturating with respect to the inhibitory effect of aurovertin on photophosphorylation: the inhibition was about 85% in both preillumated and control chromatophores, in agreement with Ref. 12. Apparently, aurovertin does not block the proton leak in preilluminated chromatophores, in contrast to oligomycin.

TABLE III

EFFECTS OF ANTIMYCIN A ON LIGHT-INDUCED PROTON UPTAKE AND PHOTOPHOSPHORYLATION IN CONTROL CHROMATOPHORES

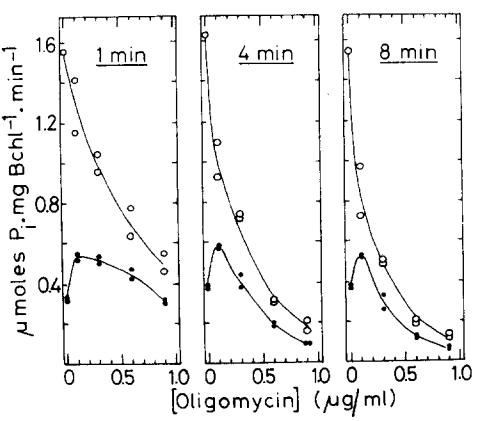
Proton uptake and photophosphorylation were assayed as described in the legends to Figs. 1 and 4, respectively, except that ethanol, or an ethanolic solution of antimycin A, was added immediately after addition of the chromatophores. Concentrations of antimycin A and oligomycin were 15 nM and 1.8 μg/ml, respectively.

	Proton uptake ($\mu M\ H^+ \cdot mg^{-1}$ Bchl)		Photophosphorylation ($\mu M\ P_i \cdot mg^{-1}$ Bchl $\cdot min^{-1}$)	
	— antimycin A	+ antimycin A	— antimycin A	+ antimycin A
— Oligomycin	0.78	0.65	2.43	1.36
+ Oligomycin	0.91	0.74	n.d. *	n.d. *

* n.d. = not determined.

Table III shows experiments in which control chromatophores were treated with a non-saturating concentration of the electron transfer inhibitor, antimycin A. At 15 nM, antimycin A caused 44% inhibition of succinate-supported photophosphorylation, and 17% inhibition of succinate-supported, light-induced proton uptake. The antimycin-induced inhibition of proton uptake was not relieved by oligomycin at 1.8 μg/ml. By contrast, both the rates and the extents of light-induced proton uptake were the same in preilluminated and control chromatophores at 1.8 μg/ml of oligomycin (Figs. 1 and 2). This indicates that light-induced electron transport was not significantly inhibited in preilluminated chromatophores, as compared to control chromatophores.

Fig. 3 shows the effect of oligomycin on light-induced 9-aminoacridin fluorescence quenching in preilluminated and control chromatophores.



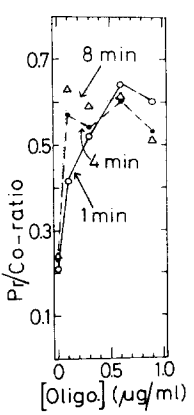


Fig. 4. Succinate-supported photophosphorylation rates in preilluminated (●———●) and control (○———○) chromatophores, as a function of the oligomycin concentration. Two samples of both types of chromatophores were assayed at each oligomycin concentration. The figure shows the phosphorylation rates measured at 1, 4, and 8 min after onset of light. The assay conditions were as described in the legend to Fig. 1, except that glycylglycine was omitted and ADP, $MgCl_2$ and sodium phosphate were present at 0.5, 3, and 3 mM, respectively.

Fig. 5. Average Pr/Co ratios of the photophosphorylation rates observed in the experiment shown in Fig. 4, at 1, 4, and 8 min after the onset of the light.

Schuldiner et al. [13] proposed that under suitable assay conditions the quenching ratio (Q/1 − Q) is proportional to the light-induced proton gradient ($[H^+]$in/$[H^+]$out) in chloroplasts, and this method has also been applied to calculate the light-induced proton gradient in chromatophores [11,14]. For this reason the steady-state extent of Q/1 − Q in the light was plotted as a function of the oligomycin concentration.

In the absence of oligomycin, the steady state extent of Q/1 − Q in the light in preilluminated chromatophores was about twice as small as in control chromatophores. At saturating concentrations, oligomycin restored the extent of Q/1 − Q in preilluminated chromatophores to about 90% of the level observed in the controls. The effect of oligomycin on the Pr/Co ratio of the extents of Q/1 − Q was half-maximal at approx. 0.25 μg/ml, and in good agreement with the results on light-induced proton uptake (Fig. 2).

Fig. 4 shows the oligomycin-dependence of succinate-supported photophosphorylation in preilluminated and control chromatophores. In control chromatophores oligomycin caused a time-dependent inhibition of phosphorylation. This occurred at all concentrations tested. In preilluminated chromatophores oligomycin had two effects on the photophosphorylation rate: A time-dependent inhibition, superimposed on a stimulation which presumably reached its full extent immediately after the onset of the light. After 1 min of illumination, the stimulation was still apparent at 0.1—0.6 μg/ml of oligomycin. At 0.1 μg/ml, oligomycin did not cause a time-dependent inhibition of phosphorylation in preilluminated chromatophores (contrast control chromatophores). From Fig. 5 it appears however, that the time-dependence of the inhibition of

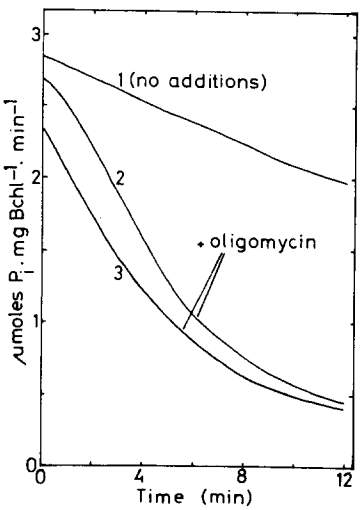


Fig. 6. Time-dependence of the photophosphorylation rate in untreated chromatophores. The curves were obtained as described in [1]. The assay conditions were as described in the legend to Table I, except that the Bchl concentration was 11 μM, phenazine methosulphate was omitted, and nigericin (1 μM) was added at 1 min after addition of the chromatophores. The light was switched on 12 min after addition of the chromatophores. Oligomycin (0.1 μg/ml) was added just prior to switching on the light (curve 2), or immediately after addition of the chromatophores (curve 3). The light was switched on at zero time.

phosphorylation by oligomycin at concentrations of 0.3 μg/ml or more, was about the same in preilluminated and control chromatophores.

The experiments shown in Fig. 6 indicate that the illumination time, rather than the contact time determined the extent of the oligomycin inhibition of phosphorylation. At 0.1 μg/ml, oligomycin caused 18% inhibition after 12 min of dark-incubation (initial parts of curves 3 and 1), likewise 18% inhibition after only 2 min of light-incubation (curve 2 vs. 1), and 77% inhibition after 12 min of light-incubation (curve 2 vs. 1). These experiments were done in the presence of nigericin in order to eliminate light-induced proton uptake. This allowed us to measure the photophosphorylation rates also during the first minute of illumination. Nigericin had no effect on the phosphorylation rates in the presence or absence of oligomycin, as measured after 1 min of illumination.

Discussion

Omission of one or more phosphorylation or arsenylation substrates during preillumination of *R. rubrum* chromatophores in the presence of phenazine methosulphate or diaminodurene, resulted in an extensive and irreversible inhibition of photophosphorylation (Tables I and II; Fig. 4) and of ATP hydrolysis (unpublished results; cf. Ref. 1). As discussed below, the inhibition of photophosphorylation was at least partly due to the presence of proton leaks in chromatophores preilluminated in the presence of phenazine methosulphate (Figs. 1—3) or diaminodurene (not shown).

The inhibition of phosphorylation by oligomycin was time-dependent. In the experiments shown in Fig. 6, the oligomycin-inhibition of phosphorylation developed 4—6 times faster during light-incubation than during dark-incubation. It made no difference in this respect, whether or not phosphorylation took place during illumination (not shown). Furthermore, the oligomycin concentration required to obtain a certain rate at which the inhibition of phosphorylation developed in the light, was in the presence of succinate plus phenazine methosulphate about 3 times lower than in the presence of succinate alone (not shown, cf. Ref. 12). All this suggests that the rate at which the oligomycin inhibition of phosphorylation develops, is energy-dependent. Membrane energization apparently leads to an, as yet unidentified, conformational or structural change in the coupling factor, which in turn is required for oligomycin-inhibition of phosphorylation to occur. More detailed mechanisms have been proposed to explain the energy dependence of the oligomycin inhibition of phosphorylation in mitochondria [15,16].

The concentrations of oligomycin required to block the proton leak in preilluminated chromatophores (Figs. 2 and 3), were in the same range as the concentrations required to inhibit phosphorylation in control chromatophores (Fig. 4). Similar results were obtained with DCCD (not shown). Like oligomycin [17—19], DCCD acts as an energy transfer inhibitor, on the level of the ATPase coupling factor in chromatophores [20,21], as well as in mitochondria [22—24]. This indicates that the proton leak in preilluminated chromatophores is located in the ATPase coupling factor. We suggest that preilluminated chromatophores contain two types of oligomycin-binding sites, viz. unmodified, or

'type 1' binding sites involved in inhibition of phosphorylation and 'type 2' binding sites associated with the proton leak. Control chromatophores, which are not leaky, contain only 'type 1' binding sites. In preilluminated chromatophores, occupation of 'type 2' binding sites by oligomycin results in closure of the proton leak and therefore in stimulation of light-induced proton uptake (Fig. 2) and of 9-aminoacridine fluorescence quenching (Fig. 3), and in stimulation of the phosphorylation rate (Figs. 4 and 5). There was no evidence for a time lag between the onset of the light and the above mentioned stimulatory effects of oligomycin (cf. Fig. 1). This indicates that 'type 2' binding sites are readily accessible for oligomycin. Compared with this, the inhibition of phosphorylation, due to oligomycin binding to 'type 1' sites, increased at a very low rate in the light (Figs. 4 and 6). The rate-limiting step may be the diffusion of oligomycin to the 'type 1' binding sites, in agreement with Ref. 15.

In preilluminated chromatophores, the oligomycin concentrations required to cause a time-dependent inhibition of phosphorylation were higher than the concentrations required to stimulate phosphorylation (Fig. 4). This may be due to competition between 'type 2' and 'type 1' binding sites for oligomycin. Since the accessibilities of 'type 1' and 'type 2' binding sites for oligomycin may be widely different, oligomycin may bind preferentially to 'type 2' binding sites when present at low concentrations. An alternative explanation may be that oligomycin inhibition of phosphorylation in preilluminated chromatophores requires a cooperative interaction between 'type 1' and 'type 2' binding sites. These two explanations are not mutually exclusive.

It should be noted that although for simplicity we have limited our discussion to oligomycin, the results shown in Figs. 1—6 were essentially the same when DCCD was used instead of oligomycin (not shown). This suggests that DCCD binds to the same sites as oligomycin does.

Succinate-supported phosphorylation was still around 40% inhibited in preilluminated chromatophores, as compared to control chromatophores, at 0.6—0.9 μg/ml of oligomycin (Fig. 4), and also at 1.8 μg/ml of oligomycin (not shown). This was not due to inhibition of light-induced electron transport in preilluminated chromatophores, as compared to controls (Table II, Figs. 1 and 2). It may, however, be at least partly due to the fact that even saturating concentrations of oligomycin closed the proton leak in preilluminated chromatophores largely, but not completely (see Fig. 3 and the data on the decay of the proton uptake in Fig. 1). In addition, phenazine methosulphate-preillumination may have induced other modifications of the coupling factor, besides the proton leak, which contribute to the residual, 40% inhibition. We do not yet know whether phenazine methosulphate-preillumination results in removal of some of the coupling factors from the chromatophore membrane. However, if at all, this occurs only on a limited state, since under certain assay conditions the uncoupler-stimulated ATPase activity of preilluminated chromatophores amounts to at least 80% of the activity of the controls (to be published elsewhere).

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