ION SENSITIVITY OF THE LIGHT REACTION CATALYZED BY ENZYME PREPARATIONS FROM *PHOTOBACTERIUM PHOSPHOREUM*

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(Received February 2nd, 1961)

SUMMARY

Cations, especially divalent ones, increase the intensity of the light flash, obtained by adding FMNH to luciferase preparations containing palmital. Besides, they cause a more rapid decay of the light reaction. Probably, the effect is due to inhibition of the oxidation of FMNH independent of the light reaction by some oxidizing substance. This substance may oxidize free as well as luciferase bound FMNH. No influence of ions on the light reaction proper could be found. The experiments suggest the conclusion that FMNH is not bound to the luciferase molecule *via* the phosphate group.

INTRODUCTION

In a previous paper[1] it was concluded that the FMNH molecule, active in the light reaction in luminous bacteria, is bound to the enzyme luciferase at two binding sites. One site of FMNH should be directly attached to the protein while the other "binding" should take place *via* a long-chain aldehyde, presumed to be attached to the luciferase. THEORELL AND NYGAARD[2] were able to draw conclusions about the mode of attachment of FMNH to the protein of Old Yellow Enzyme by measuring the influence of anions on the velocity of association and dissociation of apo-protein and prosthetic group. The light reaction in enzyme preparations of luminous bacteria is measured by adding FMNH to luciferase (+ palmital). We thus thought it possible that ions may influence the light reaction and that some conclusions may be drawn as to the mode of binding of FMNH to luciferase. THEORELL AND NYGAARD[2] found that multivalent anions decrease the association velocity of FMN and apo-protein. If FMN associates with luciferase in the same manner as in Old Yellow Enzyme, multivalent anions may be expected to decrease the initial light reaction.

Some experiments are known about the influence of ions on the light reaction. STREHLER AND CORMIER[3], using crude extracts and measuring the light reaction upon addition of DPNH found an increased light reaction if salts (NaCl, KCl, Na_2SO_4, Na_2HPO_4, $NaNO_3$, $MgCl_2$ or $CaCl_2$) at low concentration (up to about 1 %) were added. They do not offer an explanation for this result.

Non-standard abbreviations: FMN(H), (reduced) flavin mononucleotide; RF(H), (reduced)-riboflavin.

ANDERSON[4] reported a marked increase of luminescence of *Cypridina* extracts if certain salts, namely NaCl, KCl, NaBr or KBr, are added. He suggested that the salts may cause a change in the activated, light emitting, molecule which makes it less likely to loose its energy in collision with the solvent molecules.

In our experiments described below we found a marked stimulating effect of cations, especially divalent ones, on the light reaction of bacterial luciferase preparations. Probably, this effect is due to the inhibition by cations of the oxidation of FMNH outside the light reaction. An influence of ions on the light reaction proper could not be found. Some tentative conclusions about the mode of attachment of FMNH to the luciferase molecule are drawn.

MATERIAL AND METHODS

All experiments were done with preparations prepared from *Photobacterium phosphoreum*, cultivated as described earlier[1], or in the same medium containing a 3 % NaCl–KCl mixture instead of 3 % NaCl. The enzyme preparations were made following the method of MCELROY *et al.*[5] In most cases, sucrose was added until a final concentration of 0.44 *M* after lysis during 15 min. This procedure increased the activity of the preparations. The preparations obtained will be called as follows: "Lysate": the supernatant of the bacterial lysate centrifuged during 20 min at 20 000 × *g*; "HCl precipitate": the lysate proteins precipitated with 0.01 *N* HCl until pH 4.5 and redissolved in buffer; "$(NH_4)_2SO_4$ fractions 1–5": preparations obtained by adding successively 2, 1, 1, 1, 1 g $(NH_4)_2SO_4$ to 10 ml HCl precipitate and redissolving the precipitate in buffer. $(NH_4)_2SO_4$ fractions 4 and 5, containing the highest luciferase activity on a protein basis, are called "purified preparations".

The final protein concentrations, estimated by the biuret reaction with albumin as a standard, were as follows: Lysate, ± 0.5 mg/ml; HCl-precipitate, ± 1.5 mg/ml; $(NH_4)_2SO_4$ fraction 4 + 5: ± 3 mg/ml.

The preparations could be stored at —40° during 1–2 months without appreciable loss of activity.

The buffer used was Tris 0.05 *M*, pH 7.0–7.2. Owing to its ionic character, the phosphate buffer, used in former experiments, did not seem suitable.

The light reaction was evoked by adding palmital (a saturated solution in methanol, diluted as indicated) and, at zero time, FMNH to the enzyme preparation. The combination DPNH + FMN[1,6] could not be used as ions appeared to influence the DPNH-oxidase (*cf.* ref. 7). FMNH and RFH were prepared by reducing a solution of FMN (RF) with a hydrogen current until the color was light greenish yellow, using palladium black as a catalyst. Immediately after reduction, liquid paraffin was poured on top of the solution to prevent reoxidation. In some cases the FMNH solution was used without removal of the Pd through centrifugation, in other cases the Pd was centrifuged down. The FMNH (RFH) sample was taken anaerobically from the paraffin covered solution by means of a hypodermic syringe. For use, this sample was injected through a light-tight rubber stop at the bottom of the enzyme containing cuvette.

The emitted light was measured in a quantum counter by means of a liquid air cooled photomultiplier RCA 923a (see ref. 8).

The oxidation of FMNH was measured spectroscopically in a Hilger spectrophotometer. Fast reactions (Fig. 2b) were recorded by a recorder attached to the

spectrophotometer. In all anaerobic experiments the solutions used were flushed with N_2 (led over a heated copper coil) and brought under a layer of liquid paraffin in the cuvettes.

The quantities of solutions used are given with the individual experiments.

RESULTS AND CONCLUSIONS

Addition of salts to crude enzyme preparations containing palmital generally increases the total amount of light obtained by addition of FMNH (Table I). This increase depends strongly upon the valency of the cation, divalent cations in equivalent concentration being more effective than univalent ones. No significant influence of the valence of the anions (Cl^-, NO_3^-, SO_4^{2-}) was found. The accuracy of the results is not sufficient to permit the determination of an ion spectrum (*cf.* ref. 9). Thus it was not possible to obtain some information about the nature of the groups involved in this way. As Ca^{2+} ions show a clear effect all following experiments were made with $CaCl_2$.

The effect of Ca^{2+} on the light reaction might be due to an increased binding capacity of the enzyme for aldehyde in the presence of cations. If so, the Ca^{2+} effect could be expected to decrease at higher aldehyde concentrations. However, in our

TABLE I

INFLUENCE OF CATIONS ON THE INITIAL LIGHT REACTION EVOKED BY ADDING FMNH TO ENZYME PREPARATIONS CONTAINING PALMITAL

Reaction mixtures: Expts. 1, 2: 1 ml lysate, 0.3 ml aq.dest. (control) resp. salt solution, 0.01 ml palmital solution, 0.4 ml FMNH (1 mg/10 ml aq.dest.). Expts. 3, 4, 5: 1 ml lysate, 1:10, (diluted with Tris), 0.1 ml aq.dest. resp. salt solution, 0.02 ml palmital solution 1:5, 0.5 ml FMNH solution (2.5 mg/10 ml aq.dest.).

Expt. No.	*Increase of the initial light reaction (7.5 sec) in % of that of the control on addition of solutions of*						
	NaCl 0.3 M	*KCl 0.3 M*	*LiCl 0.3 M*	*$MgCl_2$ 0.15 M*	*$CaCl_2$ 0.15 M*	*$SrCl_2$ 0.15 M*	*$BaCl_2$ 0.15 M*
1	50	60	60	100	300	200	200
2	70	70	60	100	300	200	200
3	0	—	—	—	100	—	—
4	0	—	—	30	65	—	70
5	0	0	0	70	70	70	70

TABLE II

INFLUENCE OF PALMITAL CONCENTRATION ON THE INCREASE OF THE LIGHT REACTION BY Ca^{2+} IONS

Reaction mixtures: a, 0.5 ml lysate, 0.5 ml Tris (resp. 0.3 ml Tris + 0.2 ml $CaCl_2$ 0.15 *M* in Tris), 0.01 ml palmital solution 1:5, 0.5 ml FMNH (2.5 mg/10 ml aq.dest.). b, 0.1 ml lysate, 0.9 ml Tris (resp. 0.7 ml Tris + 0.2 ml $CaCl_2$ 0.15 *M* in Tris), 0.02 ml palmital solution, 0.5 ml FMNH (2.5 mg/10 ml aq.dest.).

Increase of the initial light reaction (6 sec) by Ca^{2+} at	
Low palmital concentration (a)	*High palmital concentration (b)*
150%	170%

experiments the influence of Ca^{2+} on the light production proved to be independent of the amount of palmital present in the enzyme (Table II). This result suggests that Ca^{2+} does not affect the enzyme–aldehyde linkage.

We examined whether the salt effect was also present in purified preparations. It may be mentioned here that the purified preparations are unstable as soon as they are diluted to a concentration needed in the experiment. Consequently, small differences are difficult to measure. Table III shows the influence of $CaCl_2$ addition on the light reaction of lysate, HCl-precipitate and $(NH_4)_2SO_4$ fractions 1–5. It can be seen that in most cases the salt effect is present in purified preparations. However, sometimes a preparation is obtained which either immediately after preparing or after storage at —40° does not show any Ca^{2+} effect.

TABLE III

THE INFLUENCE OF Ca^{2+} IONS ON "CRUDE" AND "PURIFIED" ENZYME PREPARATIONS

Reaction mixtures: enzyme preparation in Tris 0.9 ml, $CaCl_2$ in Tris (0.15 *M*) resp. Tris in the control 1.0 ml, palmital solution 1:5, 0.02 ml, FMNH (2.5 mg/10 ml aq.dest.) 0.5 ml.

Expt. No.	*Increase of the initial light reaction in % by Ca^{2+} in*							
	Lysate	*HCl precipitate*	*$(NH_4)_2SO_4$ Fraction 1*	*Fraction 2*	*Fraction 3*	*Fraction 4*	*Fraction 5*	*Fraction 4 + 5 2 × precipitated*
1	40	50	40	50	80	50	40	—
2	60	100	30	60	40	> 100	100	—
3	—	—	—	—	—	70	80	—
4	—	—	—	—	—	100	0	—
5	—	—	—	—	—	—	—	50
								After 7 days 0
6	140	—	—	—	—	—	—	30

The absence of the salt effect in a number of purified preparations might be due to the loss of some inhibitor, activity of which is decreased by Ca^{2+} ions. In our previous experiments[1], the existence of an isooctane-extractable inhibitor in bacterial enzyme preparations was indicated. Thus, we examined whether or not the influence of salts was lost upon isooctane extraction of the enzyme. This proved not to be the case. Consequently, the isooctane-extractable inhibitor is not responsible for the salt effect.

The presence of NH_4^+ and SO_4^{2-} ions in purified preparations might be another cause for the decrease of the Ca^{2+} effect. Therefore, the salt concentration was diminished by dialyzing the preparation against distilled water. However, this procedure annihilated the enzyme activity. It could not be restored, neither by salt addition nor by addition of the concentrated dialyzate, so no definite conclusions could be drawn from these experiments.

The increased light reaction in enzyme preparations in the presence of $CaCl_2$ consists of a higher initial flash after addition of FMNH. In many experiments, it was observed that at the same time the light intensity decreases more rapidly. A typical experiment is shown in Fig. 1. If there is a continuous supply of FMNH, which is the case if FMNH is replaced by a mixture of DPNH + FMN, a continuous light reaction is observed instead of a flash. Consequently, the decay of the light intensity, using FMNH, will be due, at least for the greater part, to the oxidation of FMNH. In

order to examine whether Ca^{2+} influences the oxidation of FMNH this oxidation was followed spectroscopically by measuring the velocity of appearance of the 445-mμ absorption band of FMN. If FMNH, containing some Pd–H_2, is injected in a cuvette containing a similar reaction mixture as in the light reaction (without palmital, with or without Ca^{2+}) a total oxidation of FMNH, within 1 sec, was observed. The presence of palmital did not have any perceptible influence on the oxidation velocity of FMNH. Probably, the oxidation of FMNH is due to the O_2 present in the preparation. There-

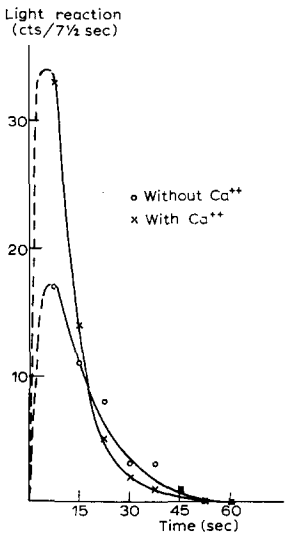

Fig. 1. The light reaction decay curve with and without Ca^{2+} ions added to the enzyme preparation. Reaction mixtures: 1.0 ml enzyme lysate, 0.3 ml aq.dest. resp. $CaCl_2$ 0.015 *M*, 0.02 ml palmital solution 1:5, 0.5 ml FMNH (1.0 mg/10 ml aq.dest).

fore, the FMNH solution, containing some Pd–H_2, was introduced into the spectrophotometer cuvette under a paraffin layer and buffer, flushed with pure N_2 during 20 min, was added. In this way only a very slight oxidation of FMNH occurred. However, with enzyme preparations (lysate), flushed with N_2 in the same way, instead of buffer, a rapid oxidation of FMNH, within 1 sec, took place under anaerobic conditions. Addition of $CaCl_2$ to the enzyme preparation decreased the oxidation of FMNH. The oxidation of FMNH was followed by a slow back reduction, probably caused by the Pd–H_2 present in the FMNH solution. Owing to the rapid oxidation of FMNH during the injection procedure, working without Pd–H_2 appeared not to be possible in these experiments. In Fig. 2 the results of some typical experiments are shown. It was extremely difficult to add the enzyme or buffer very rapidly to the paraffin covered FMNH while maintaining rigorously anaerobic conditions. Therefore, in most experiments the enzyme was added carefully to the FMNH immediately before placing the cuvette in the spectrophotometer and measuring the absorption (Fig. 2a). However, the initial effect is not registered in this way. Some experiments were made with a recorder attached to the spectrophotometer. The curves obtained (Fig. 2b) give an indication of the initial effect. In these experiments a considerable part of FMNH is oxidized by traces of O_2. However, the results enable us to draw the dashed parts of the curves in Fig. 2a.

The oxidation of FMNH and the Ca^{2+} effect were also observed with purified preparations.

From these experiments we conclude that, in the enzyme preparations, a FMNH oxidizing substance is present. The oxidation reaction is inhibited by Ca^{2+}. The oxidation of FMNH and the Ca^{2+} effect are also measurable in enzyme preparations that have been heated during 10 min in a water bath at 100°. Consequently, the oxidant is thermostable.

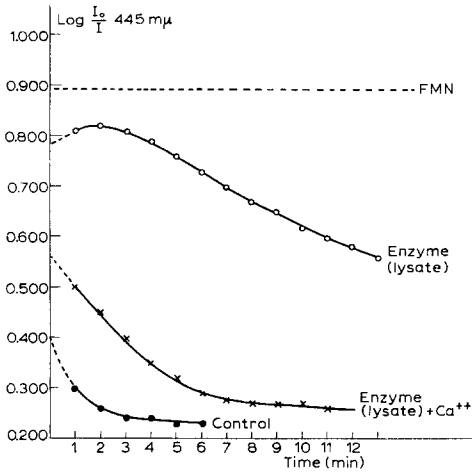

Fig. 2a.

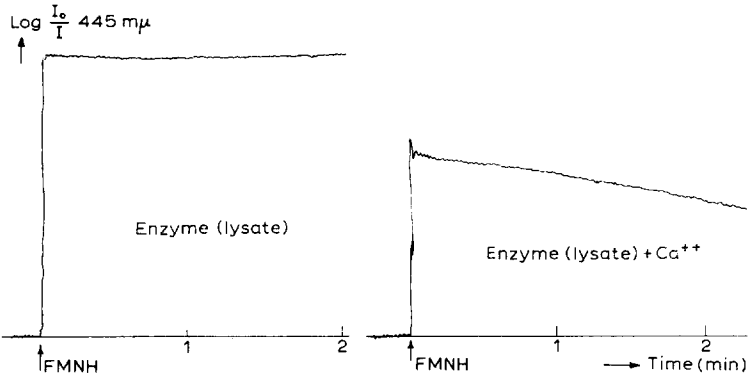

Fig. 2b.

Fig. 2. The oxidation of FMNH by enzyme and enzyme + Ca^{2+}. (a) Backreduction curves of FMN (the enzyme preparation is carefully added to FMNH). (b) The initial effect (the enzyme preparation is very rapidly added to FMNH). Reaction mixtures: 0.9 ml enzyme lysate, 0.1 ml Tris resp. 0.15 *M* $CaCl_2$ in Tris, flushed with N_2, 0.5 ml FMNH (1 mg/10 ml aq.dest.), containing Pd–H_2.

It was shown that under the aerobic circumstances of the light reaction added FMNH is oxidized within 1 sec. However, under the conditions of most experiments, the light emission is perceptible for at least 20 sec. Considering the anaerobic oxidation experiments with FMNH, there are two possible explanations for this apparent discrepancy: (a) FMNH is oxidized by both O_2 and a substance X. The reduced form

of the latter, XH, participates in the light reaction. (b) FMNH is oxidized by O_2 and by X; XH is inactive in the light reaction. The remaining amount of FMNH, active in the light reaction and perhaps bound to the enzyme, is too small to be detected spectroscopically.

As Ca^{2+} appears to inhibit FMNH oxidation and Ca^{2+} increases the light reaction, the second explanation seems, so far, the most probable one.

Considering these experiments, it might be supposed that the only effect of Ca^{2+} is the inhibition of the oxidation of FMNH by some substance in the enzyme preparation. If so, Ca^{2+} addition would result in an increase in the amount of substrate, FMNH, for the enzyme reaction. This does not explain, however, why the decay of the light-reaction is enhanced in the presence of Ca^{2+}. The latter effect suggests an activation of the enzyme by Ca^{2+}. The results of the experiments described below provided an "explanation" for this enzyme-activating action of Ca^{2+}.

It was thought probable that the phosphate groups of FMNH are essential for the Ca^{2+} effect in the oxidation reaction. Therefore, a number of experiments with riboflavin were made. It was found already by HASTINGS AND MCELROY[10] that riboflavin is an inhibitor of luminescence in bacterial extracts. However, the presence of FMN also causes a decrease of the light reaction (competition with FMNH?) STREHLER *et al.*[11] found that reduced riboflavin causes a light flash if added to bacterial enzyme preparations. MCELROY AND GREEN[12] stated that RFH is active only if FMN is present in the preparation. These observations could be confirmed. It was also established that the light reaction using RFH + FMN lasts longer than that with FMNH. This is caused, probably, by a slower autooxidation of RFH. The above makes us assume, as did MCELROY AND GREEN[12], that RFH is active only because it reduces FMN. However, considering the oxidation–reduction potentials of both substances (FMN —0.190 V, RF —0.185 V at pH 7.0, *cf.* VESTLING[13]) this explanation does not

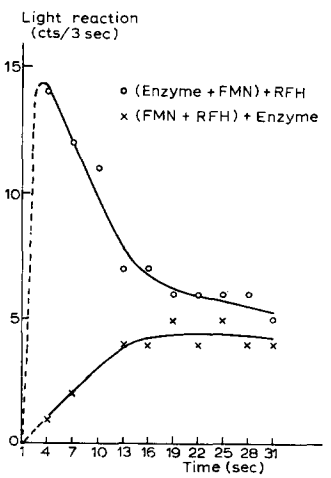

Fig. 3. The initial light reaction of an enzyme preparation added to a mixture of FMN and RFH compared with the flash reaction evoked by adding RFH to an enzyme preparation containing FMN. Reaction mixtures: 0.4 ml Tris, 0.1 ml FMN (2.5 mg/10 ml aq.dest.), 0.02 ml palmital solution 1:5. O—O, At t = 0 sec 0.5 ml enzyme preparation (doubly precipitated fraction 4 + 5 in Tris) is added and at t = 1 sec 0.5 ml RFH (± 2 mg/10 ml aq.dest.). ×—×, At t = 0″0.5 ml RFH and at t = 1″0.5 ml enzyme preparation is added.

seem entirely satisfying. Besides, we found that no immediate light flash is obtained if RFH and FMN are mixed and the enzyme is added afterwards (Fig. 3), indicating that at mixing RFH and FMN without enzyme preparation no FMNH is formed. If RFH is added to a mixture of enzyme FMN an immediate light flash is observed. The same result is obtained if FMNH is added to enzyme + RF. We conclude from these experiments that FMN can be reduced because it is bound to the enzyme. This binding may cause an increase in the oxidation–reduction potential (*cf.* ref. 13).

Upon anaerobic addition of enzyme preparation to RFH (+ PD–H_2) no or only very little oxidation of RFH was observed spectrophotometrically. Apparently, the substance X is not readily reduced by RFH. Ca^{2+} did not have a detectable influence. These experiments suggest that the phosphate group of FMNH is essential for the oxidation reaction and the Ca^{2+} effect.

The influence of Ca^{2+} on the light reaction, initiated by adding RFH to a preparation containing FMN is substantially the same as that on the light reaction initiated by adding FMNH. From this observation we conclude that Ca^{2+} also influences the oxidation of FMNH formed by reduction by RFH, *i.e.* it influences the oxidation of enzyme-bound FMNH.

In our scheme of the light reaction[1,6] the palmital is assumed to be attached to the luciferase very close to the bound FMNH. If the FMNH molecule near the palmital is oxidized outside the light reaction by the Ca^{2+}-inhibited oxidizing substance, the palmital situated next to the FMNH cannot be active in a light-emitting oxidation reaction of FMNH. Thus, addition of Ca^{2+} will, besides increasing the amount of substrate, increase the enzymic activity.

DISCUSSION

The present experiments suggest the following conclusions about the binding of FMNH to the luciferase.

RF inhibits the light reaction. This is interpreted to indicate a competition between RF and FMN for the same site at the enzyme[11,14]. The binding group will be common to the two molecules, it might be the iso-alloxazine part of the molecule or the ribityl group[14].

In the Old Yellow Enzyme the phosphate group of FMN is presumed to be bound to NH^+ groups of the apo-protein. If FMNH were attached to luciferase in this way, a light inhibiting effect might be expected to result from the addition of salts, the ions screening the ionized groups.

WALAAS AND WALAAS[15] demonstrated an inhibition of the activity of D-amino acid oxidase, a flavoprotein, by different anions. The experiments are interpreted to indicate that the phosphoric ester groups of the flavin adenine nucleotide molecule are of importance in the coupling to the apo-enzyme.

An inhibiting effect of salts on luciferase activity could not be found. We suggest, therefore, that the phosphate group of FMNH is not bound to the luciferase.

Ca^{2+} ions appear to inhibit the oxidation of FMNH outside the light reaction. The FMNH-oxidizing substance is thermostable. Besides, some active preparations with respect to the light reaction were obtained that did not show the Ca^{2+} effect. We concluded, therefore, that the Ca^{2+} effect is due to some substance generally accompanying luciferase in our preparations, but not essential for the light reaction.

It was concluded that Ca^{2+} acts on enzyme bound FMN too. As the phosphate group of FMN is supposed not to be bound to the luciferase and as the presence of the phosphate group is essential for the oxidation by some substance X it may be suggested that Ca^{2+} inhibits the reaction between FMNH, whether or not in the bound form, and the oxidizing substance X by screening the free phosphate group of FMNH.

We assume that during the light reaction FMNH is bound to the enzyme *via* the iso-alloxazine ring system or *via* the ribityl group[14], but not *via* the phosphate group. In a previous investigation[16] we suggested that FMN is also "bound" to the enzyme *via* the long-chain aldehyde. It is not possible to draw any conclusions about this "bond" from the experiments described above.

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