THE PHOTOINHIBITION OF MALATE DEHYDROGENASE

G.A. CODD

Max-Planck-Institut für Züchtungsforschung (Erwin-Baur-Institut), Abteilung Menke, 5 Köln, Germany

Received 22 November 1971

1. Introduction

Recently several enzymes which require FMN as a cofactor have been shown to be inhibited by blue light in the presence of added FMN [1–3]. Photoinactivation of the enzyme protein occurred via the formation of triplet FMN. The present paper reports, for the first time, an FMN-mediated photoinhibition of NAD-malate dehydrogenase, a key enzyme of the Krebs Cycle, at low light intensities. Evidence is presented that a binding site for FMN must exist on this enzyme.

2. Materials and methods

MDH (L-malate: NAD oxidoreductase EC 1.1.1.37) from pig heart mitochondria was purchased from Boehringer (Mannheim). *Euglena gracilis* Klebs Z was grown phototrophically on 5% CO_2 in air and an extract containing MDH prepared as described previously [4]. Leaves from 6–8 week old *Nicotiana tabacum* var. John Williams' Broadleaf grown according to Schmid [5] and from 11 day old *Zea mays*, cultured in sand in a greenhouse, were washed and homogenised for 1 min at 4° in a Waring blendor in twice their weight of 0.1 M phosphate buffer, pH 7.0. The brei was filtered and the filtrate centrifuged as before [4] and the supernatant solutions used. Thin-walled tubes containing 0.05 mg pig heart MDH protein or between 2–5 mg of plant protein, and 100 μmoles of buffer, and other additions in 2.0 ml as detailed in the Results, were incubated in a water bath at 37 or 25°. Incubations were performed in the dark or in red or blue light. Light sources, filters and the integration of transmitted energy were all as described earlier [1].

Assays were performed at room temp in 3 ml quartz cuvettes of 1 cm light path, containing 250 μmoles buffer, 0.025–0.1 ml enzyme incubation mixture, 1 μmole NADH and water to 2.5 ml. The reaction was started by adding 5 μmoles oxalacetate and the decrease in absorbance at 334 nm measured on an Eppendorf S 110-E spectrophotometer with a model 4410 recorder. Protein was determined by the Lowry method [6]. N, N, N′, N′-tetramethyl-p-phenylene-diamine (TMPD) was purchased from Schuchardt (München) and β-carotene from Fluka (Buchs, Switzerland). 3-(chlorophenol)-1, 1-dimethylurea was a gift from Prof. A. Trebst, University of Bochum.

3. Results and discussion

3.1. *Animal MDH*

Incubation at the optimum pH of 8.5 in blue light alone did not affect activity, however the presence of even low concentrations of FMN resulted in marked inhibition (fig. 1). The FMN-dependent photoinhibition occurred throughout the enzyme's pH range. Exposure to 1542 ergs/cm^2/sec of blue light (i.e. approx. 300 lux) with 5×10^{-7} moles FMN gave a 47.5% inhibition after 30 min (fig. 2). Indeed, a 37% inhibition was observed after 30 min in the presence of blue light of only 236 ergs/cm^2/sec (about 47 lux).

Illumination by red light (22,600 ergs/cm^2/sec) for 2 hr with and without FMN did not affect enzyme activity. Exhaustive dialysis of blue light-inhibited enzyme to remove FMN did not result in a restoration of activity, suggesting the inhibition to be irreversible. FMN was illuminated aerobically and anaerobically alone and with an equimolar amount of glutamic acid. The additions of photoproducts thus produced

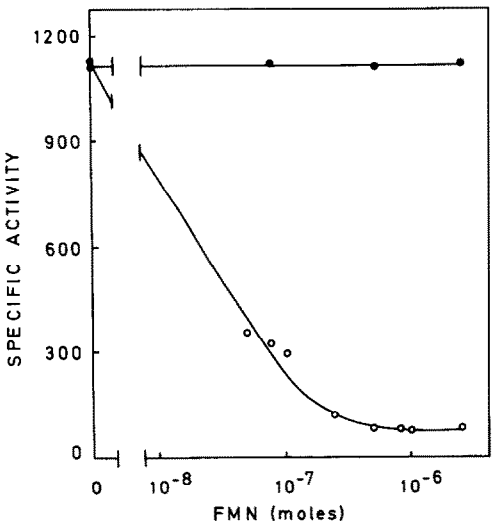

Fig. 1. The effect of blue light and FMN on malate dehydrogenase. Activity was determined after aliquots of enzyme, containing 0.05 mg protein, were incubated in the dark or in blue light (1542 ergs/cm^2/sec; 380 <λ< 575 nm) in 0.1 M phosphate buffer, pH 8.5 at 37° for 2 hr (final vol 2.0 ml). FMN was added in the amounts shown on the abscissa. ●—● : dark; ○—○ : blue light. Specific activity: μmoles NADH oxidised/min/mg protein.

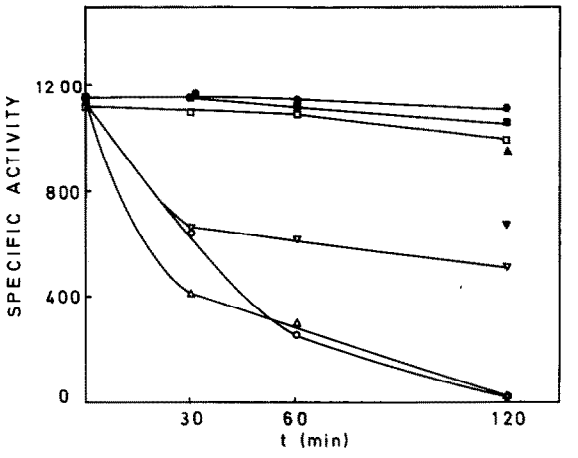

Fig. 2. Blue light inhibition of malate dehydrogenase in the presence and absence of oxygen. Incubation conditions were as described in fig. 1 at pH 8.5, except that Warburg vessels were used. ■—■: dark; ●—●: dark plus FMN; ▲: blue light plus O_2; ▼: blue light plus O_2-free N_2; □—□: blue light in air; ○—○: blue light + FMN in air; △—△: blue light plus FMN in O_2; ▽—▽: blue light plus FMN in O_2-free N_2. t: incubation time.

Table 1
The quenching of FMN-mediated blue light inhibition of malate dehydrogenase by various compounds [a].

Added compound	Blue light 380 <λ< 575 nm 1542 ergs/cm^2/sec	Relative rate [b]
None	Dark	100.0
None	Blue light	8.0
Tryptophan	Dark	91.5
Tryptophan	Blue light	64.5
CMU	Dark	76.4
CMU	Blue light	49.0
β-carotene	Dark	96.0
β-carotene	Blue light	18.8
Hydroquinone	Dark	0.9
Hydroquinone	Blue light	1.7
TMPD	Dark	73.3
TMPD	Blue light	63.9

a: 1μmole of compound was added to the incubation mixture and activity measured after 2 hr at 37°, pH 8.5 in the presence of 5×10^{-7} moles FMN (final vol 2.0 ml).
b: Results are presented as relative to the rate measured with dark-treated enzyme.

[7] to the enzyme did not significantly affect activity, showing that association or attachment of ground state FMN to the enzyme may be a prerequisite for photoinhibition. It follows that a binding site for FMN must occur on this enzyme.

It is widely recognised that, with few exceptions, flavin-sensitized photoreactions proceed via the triplet state of the sensitizer [1, 7, 8]. As reviewed by Foote [9] subsequent energy transfer from triplet flavin may proceed by 2 types of reaction, namely by interaction with a substrate (which in this case would be MDH) or by interaction with oxygen. The route involving oxygen may proceed either by formation of a dye peroxide and subsequent reaction with a reductant (oxygen transfer), or via singlet oxygen formation. These possibilities were tested by measuring photoinhibition in the presence and absence of oxygen, a triplet quencher of FMN [9], and of other triplet- and singlet quenchers.

The quenching effects of tryptophan, a quencher of triplet–triplet transfer in FMN [8], and of CMU, which forms a complex with triplet flavin [10], clear-

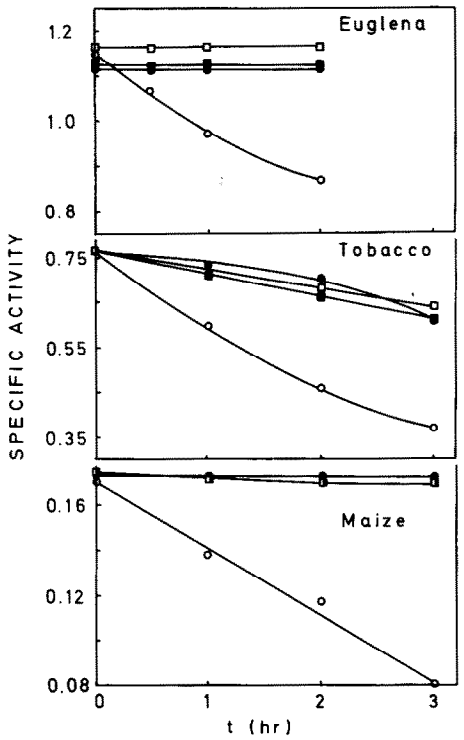

Fig. 3. The inhibition of plant NAD-malate dehydrogenase by blue light in the presence of FMN. Cell-free extracts of *E. gracilis*, *N. tabacum* and *Z. mays*, prepared as described in Materials and methods, were incubated in 0.1 M phosphate buffer pH 8.5 at 25° either in the dark or in blue light (1542 ergs/cm^2/sec), both with and without 5 × 10^{-7} moles FMN (final vol 2.0 ml). ■–■: dark; ●–●: dark plus FMN; □–□: blue light; ○–○: blue light plus FMN. Specific activity: μmoles NADH oxidised/min/mg protein

ly show that the primary photoact consists of triplet FMN formation (table 1). The effect of oxygen availability was determined by incubating the enzyme in Warburg flasks with 100% oxygen or *oxygen-free* nitrogen. If inactivation proceeded exclusively via the quenching of triplet FMN by substrate (MDH), then the inhibition should be quenched by paramagnetic oxygen. Incubation under nitrogen should prevent inhibition if it proceeded only via singlet oxygen. Fig. 2 suggests that both mechanisms are involved since the final degree of inhibition was not significantly affected by pure oxygen, but was quenched by about 50% under nitrogen. β-carotene and hydroquinone act as singlet oxygen quenchers [11, 12]. Hydroquinone almost completely inhibited the enzyme in the light and dark showing that MDH has a site (s) susceptible to quinone oxidation. However, the low amounts of β-carotene, dissolved in ethanol, caused quenching of the photoinhibition (table 1). TMPD also quenched the inhibition (table 1). The effect of TMPD is in accordance with the findings of Ouannès and Wilson that certain amines quench singlet oxygen [13].

Thus under the conditions employed, the photoinactivation mechanism is visualised as proceeding via 2 routes. In each case, the primary photoact consists of the excitation of FMN to the triplet state. Inactivation may then occur by energy transfer from triplet FMN to a substrate or via singlet oxygen formation. The fact that photoinhibition, though on a reduced level, occurred under oxygen-free nitrogen further suggests that both routes may be concurrently employed. As discussed previously [9], which of the 2 routes predominates will depend upon several factors, including relative oxygen and substrate concentrations and reaction rates of triplet FMN with substrate and oxygen.

3.2. *Plant MDH's*

MDH preparations from *E. gracilis*, *N. tabacum* and *Z. mays* were inhibited in blue light plus low concentrations of FMN (fig. 3). It is probable that the plant preparations contained triplet- and singlet-quenching compounds and purification may result in higher inactivation rates. Nevertheless the blue light effect was readily observed within 30 min of illumination (fig. 3). The photoinhibition of NAD–MDH in illuminated plant tissue will depend upon relative concentrations of enzyme and biological sensitizing and quenching compounds. The paper shows that a direct effect of light on a respiratory enzyme is possible.

Acknowledgements

The author thanks Dr. G.H. Schmid for helpful discussions and Frau P.-M. Lürssen for preparing the figures. The work was performed during the tenure of a fellowship of the Max-Planck-Gesellschaft.

References

[1] G.H. Schmid, Hoppe-Seylers Z. Physiol. Chem. 350 (1969) 1035.
[2] G.H. Schmid and P. Schwarze, Hoppe-Seylers Z. Physiol. Chem. 350 (1969) 1513.
[3] G.H. Schmid, Hoppe-Seylers Z. Physiol. Chem. 351 (1970) 575.
[4] G.A. Codd, J.M. Lord and M.J. Merrett, FEBS Letters 5 (1969) 341.
[5] G.H. Schmid, Planta 77 (1967) 77.
[6] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[7] J. Posthuma and W. Berends, Biochim. Biophys. Acta 112 (1966) 422.
[8] G.K. Radda, Biochim. Biophys. Acta 112 (1966) 448.
[9] C.S. Foote, Science 162 (1968) 963.
[10] P. Homann and H. Gaffron, Photochem. Photobiol. 3 (1964) 499.
[11] C.S. Foote and R.W. Denny, J. Amer. Chem. Soc. 90 (1968) 6233.
[12] C.S. Foote, R.W. Denny, L. Weaver, Y. Chang and J. Peters, Ann. New York Acad. Sci. 171 (1970) 139.
[13] C. Ouannès and T. Wilson, J. Amer. Chem. Soc. 90 (1968) 6527.