ON THE FORMATION OF THE TPN REQUIRING GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE DURING THE PRODUCTION OF CHLOROPLASTS IN *EUGLENA GRACILIS*

GEORGE BRAWERMAN AND NANCY KONIGSBERG

Cell Chemistry Laboratory, Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, N.Y. (U.S.A.)

(Received March 1st, 1960)

SUMMARY

Green Euglena cells possess a TPN-requiring glyceraldehyde phosphate dehydrogenase, in addition to the DPN-requiring enzyme. The dark-grown, colorless cells contain only the DPN enzyme. In resting colorless cells exposed to light, the course of formation of the TPN enzyme follows closely that of chlorophyll. Removal of light during this process results in a cessation of both enzyme production and chlorophyll synthesis. Cultivation of the green cells at 34° in the light produces an inhibition of chloroplast synthesis, and return of the cells to a lower temperature brings about a rapid resumption of chloroplast synthesis after a lag period of one to two days. The production of the TPN enzyme follows very closely that of the chloroplasts, both during the inhibition and the recovery. The synthesis of the DPN enzyme, on the other hand, is linked to the multiplication of the cells during this process.

INTRODUCTION

Green plant tissues have been found to contain a GPDase that requires TPN[1,2]. This enzyme is absent from the non-photosynthetic plant tissues, which contain the DPN dependent enzyme. The TPN activity has been shown further to be the result of two different enzymes; one which requires inorganic phosphate or arsenate, and another one that does not have these additional requirements[3,4]. HAGEMAN AND ARNON have studied the formation of the TPN enzymes during the germination of pea seeds[5]. The appearance of the TPN dependent activity in the shoot was found to occur only in the presence of light. This process thus appears to be analogous to the induced enzyme formation in bacteria.

In conjunction with studies in this laboratory of the chemical events accompanying the formation of the photosynthetic apparatus in dark-grown (colorless) *Euglena gracilis* cells exposed to light[6,7], it appeared of interest to follow the emergence of a specific enzyme connected with photosynthesis. The TPN linked GPDase has

Abbreviations: GPDase, glyceraldehyde-3-phosphate dehydrogenase; TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; FDP, fructose-1,6-diphosphate; G6P, glucose-6-phosphate.

been reported to be present in the green *Euglena gracilis* cells and absent from the colorless and the permanently bleached organisms[8]. We have studied the production of this enzymic activity during the formation of chloroplasts in *Euglena*. The use of a unicellular photosynthetic organism has enabled us to study the formation of this enzyme under strictly controlled conditions, and has revealed a very tight regulation of enzyme production during the synthesis of the chloroplasts.

EXPERIMENTAL

Materials

Fructose-1,6-diphosphate, Mg salt, and cysteine hydrochloride were purchased from Schwarz Laboratories, Mount Vernon, N.Y.; DPN, TPN, and glucose-6-phosphate, Na salt, from Sigma Chemical Co., St. Louis; DL-glyceraldehyde-1-bromide-3-phosphoric acid, dioxane complex, from California Corporation for Biochemical Research, Los Angeles; crystalline aldolase from Nutritional Biochemical Corporation, Cleveland.

Cultures: Euglena gracilis, strain z was used. The broth and "resting" media have been described previously[6].

Preparation of extracts

The cell samples were collected by centrifugation, washed with water and stored in a freezer. The following extraction procedure, adapted from that used by HAGEMAN AND ARNON[5], yielded reproducible and presumably quantitative amounts of enzyme from the cells. All the operations were carried out in the cold room. Acetone was cooled to the temperature of a dry ice–ethanol bath, then added to the samples. The frozen cell mass was disrupted by vigorous stirring until a homogeneous suspension in the acetone was obtained. The material was then collected on a small Buchner funnel and suction was continued for a few minutes in order to eliminate the residual acetone. The resulting dry powder was transferred into a centrifuge tube. At this stage, the material could be stored for a few days in the freezer. The active extracts were obtained by suspending the acetone-treated samples in a 0.02 *M* NaCl–0.0015 *M* ethylenediaminetetraacetate, pH 6.4, solution, stirring the suspension for 1 min, then centrifuging. The GPDase activities in the supernatant were rather unstable, and the assays had to be done within 15 min after the final extraction. It was found later that addition of neutralized cysteine hydrochloride to the extracts improved the stability of the enzymes.

Enzyme assays

FDP in conjunction with aldolase was used as the substrate for the GPDase measurements[9]. The assay mixture consisted of 0.07 *M* tris (hydroxymethyl) aminomethane, pH 8.5, 0.007 *M* cysteine hydrochloride, 0.007 *M* Na arsenate, 60 μg/ml of aldolase, 70 μg/ml of TPN, 0.008 *M* FDP, and extract in a total volume of 3 ml. DPN was substituted for TPN when the DPN requiring enzyme was determined, and FDP was replaced by G6P in the assay for G6P dehydrogenase. The assay mixture minus FDP was incubated with the extract for about 5 min; a blank without extract was also incubated. The reaction was started by adding FDP to both the test mixture and the blank, and 1 min later the increases in O.D. at 340 mμ were measured every

30 sec for 2 min. The activity was expressed as the increase in O.D. units/min. The rate of increase in O.D. was usually constant for about 5 min in the case of the TPN-requiring enzyme. With the DPN enzyme, however, this rate decreased rapidly, and the measurements were recorded for only 1.5 min. The activity was fairly proportional to the amount of enzyme in the range of 0.01 to 0.05 O.D. units, and the amounts of extracts used for the assay were adjusted to give activities within this range. The test as used does not distinguish between the two different TPN requiring GPDases, and the results obtained therefore represent the overall TPN-GPDase activity.

Cell fractionation

One volume of 10% sucrose in 0.01 *M* Tris buffer pH 7.6 and 0.005 *M* $MgCl_2$ was added to an equal volume of packed cells, freshly collected. The cell suspension was treated in the cold for 3 min in a glass tissue grinder equipped with a tight fitting teflon pestle. Six volumes of the sucrose solution were then added to the disrupted cells and the suspension was centrifuged at 1000 × *g* for 20 min. The sediment, containing both chloroplasts and unbroken cells, was resuspended in fresh sucrose solution and the cells were removed by repeated centrifugations at 140 × *g* for 2 min. The chloroplasts were then resedimented at 1000 × *g* for 20 min. The supernatant from this last centrifugation ("chloroplast-supernatant") was saved. The combined mitochondria and microsomes were obtained by centrifugation of the first supernatant at 100,000 × *g* for 60 min. The resulting supernatant was used as "soluble fraction". All fractions were stored in the freezer. This fractionation procedure yielded apparently intact chloroplasts. Some plastid breakage occurred,as suggested by the presence of chlorophyll in the 1000 × *g* supernatant.

For the enzyme assays, the particulate fractions were either treated with cold acetone before extraction, or extracted without any acetone treatment. The latter procedure gave somewhat higher enzyme activities.

Analytical methods

For protein measurements, cell samples were successively extracted with cold 5% trichloroacetic acid and with ether–alcohol (3:1), dissolved in 0.1 *N* NaOH and determined by a biuret method[10]. Cell counts were done in a hemocytometer after immobilization with a little chloroform. Chlorophyll was determined by O.D. measurements at 665 mμ of ethanol extracts[6].

RESULTS

Enzymic activities in green and colorless cells

The use of FDP as substrate with crude extracts of plant tissues could lead to the formation of G6P and subsequent reduction of TPN with the help of G6P dehydrogenase[11]. The fact that G6P dehydrogenase does not require sulfhydryl groups makes the estimation of this side reaction possible. The results in Table I indicate that the extracts from green cells exhibit very little activity with FDP as the substrate in the absence of cysteine. The reduction of TPN by G6P, on the other hand, is completely independent of cysteine. The small activity obtained with FDP and TPN in the absence of cysteine could therefore be a measure of the contaminating G6P dehydro-

TABLE I

GLYCERALDEHYDE-3-PHOSPHATE AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITIES OF GREEN AND COLORLESS *Euglena* CELLS

Activities expressed as O.D. units/mg of total cell protein.

Type of cells	*Substrate*	*Cofactor*	*Activity*	
			No cysteine	*Cysteine added*
Green	FDP	TPN	0.011	0.083
	FDP	DPN	0.005	0.43
	G6P	TPN	0.026	0.025
Colorless	FDP	TPN	0.012	0.015
	FDP	DPN		0.53
	G6P	TPN		0.015

genase activity, and the assay method used in this investigation appears sufficiently specific for the measurement of the TPN requiring GPDase activity. As a verification, FDP and glyceraldehyde-3-phosphate were compared as substrates, and similar values for the rates of reduction of TPN were obtained*. In the colorless cells, the TPN activity is essentially independent of cysteine, and is equal to the cysteine independent activity of the green cells. This most likely does not represent GPDase activity.

The results of Table I indicate that the amount of DPN dependent GPDase activity of the green cells is 5 to 6 times higher than the corresponding TPN activity. The DPN activity of the colorless cells appears slightly higher than that of the green cells.

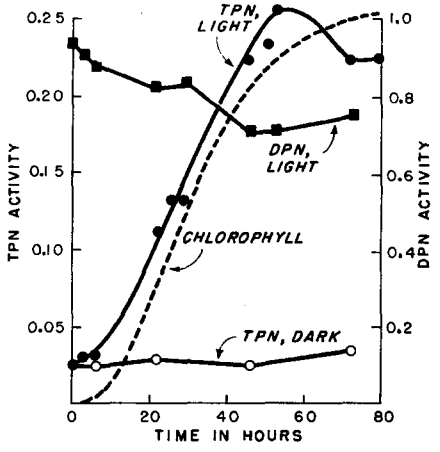

Fig. 1. Glyceraldehyde-3-phosphate dehydrogenase activities during the formation of chloroplasts in resting colorless cells. Cells from 2-l culture grown in the dark collected, suspended in 400 ml resting medium. One-half of suspension exposed to light, (full circles); other portion kept in the dark (open circles). 20-ml samples collected for the enzyme assays. Chlorophyll (broken line) determined on separate samples. TPN activity: circles; DPN activity: squares.

* HAGEMAN AND ARNON, in their study of the GPDase activity in germinating pea seeds, also concluded that FDP was a satisfactory substrate for their extracts[5].

Appearance of TPN glyceraldehyde-3-phosphate dehydrogenase during the formation of chloroplasts in colorless cells

The course of formation of the TPN GPDase activity in resting colorless cells exposed to light is described in Fig. 1. The formation of the TPN enzyme follows closely the production of chlorophyll in the cells. The small drop in activity near the completion of the greening process was encountered in most experiments. The DPN activity decreases somewhat during the process.

Removal of the light source before the completion of the greening process results in an immediate cessation of chlorophyll synthesis[12]. The production of the TPN GPDase activity is also blocked when the cells are returned to the dark (Fig. 2).

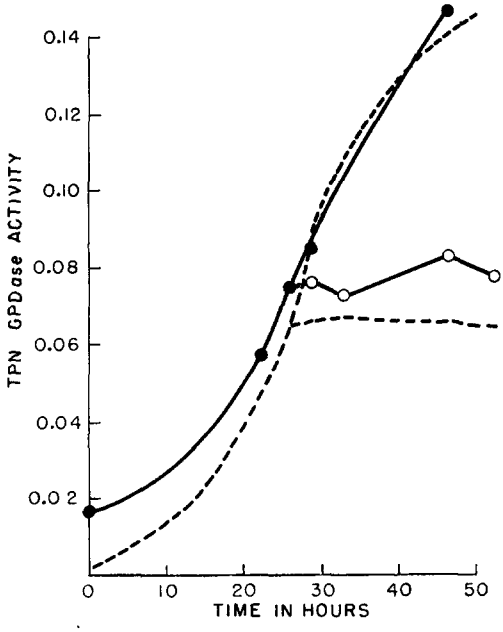

Fig. 2. Effect of removal of light on TPN glyceraldehyde-3-phosphate dehydrogenase production in colorless cells. Experimental conditions similar to those in Fig. 1. After 26 h in the light, part of the suspension returned to the dark. Full circles: cells in the light; open circles: cells returned to the dark; broken line: chlorophyll.

Regulation of the formation of the TPN-dependent glyceraldehyde-3-phosphate dehydrogenase during the bleaching treatment

It has been shown previously that growth of the *Euglena* cells at 34.5° (bleaching temperature), which brings about the permanent loss of their ability to form chloroplasts, causes a disproportionation between the multiplication of cells and the production of plastids[12]. This results in organisms containing progressively fewer chloroplasts. We have followed the formation of both the TPN and the DPN enzymes during this process. Under non-growing conditions, the bleaching temperature causes only a slight retardation in the development of chloroplasts in colorless cells. Similarly its effect on the formation of the TPN enzyme is very small (see Table II). In Fig. 3, the course of events during growth at the bleaching temperature is described. The rise in the TPN enzyme follows very closely the formation of chlorophyll. When the

TABLE II

EFFECT OF THE BLEACHING TEMPERATURE OF THE FORMATION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE IN RESTING COLORLESS CELLS

Cells from 1 l of dark grown culture collected, washed, suspended in 300 ml resting medium. One portion kept at 34.5°, the other one at room temperature. Both suspensions kept in the light.

Time (h)	*Chlorophyll*		*TPN GPDase activity*	
	Room temperature	*Bleaching temperature*	*Room temperature*	*Bleaching temperature*
0	0.007	0.007	0.03	0.03
21	0.50	0.37	0.14	0.12
47	0.74	0.75	0.19	0.22

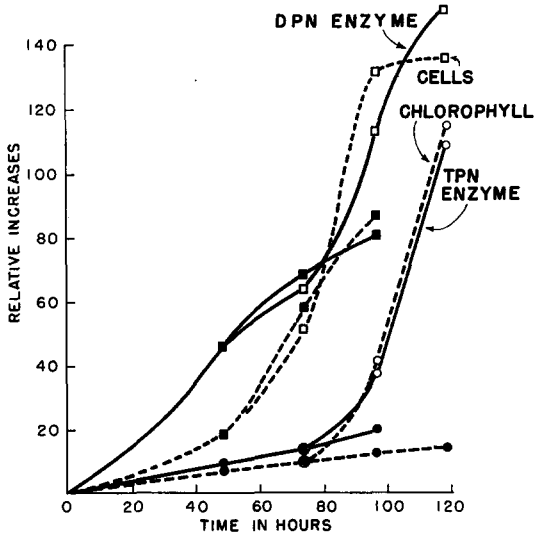

Fig. 3. Formation of the glyceraldehyde-3-phosphate dehydrogenases during the bleaching treatment. 6 ml green culture inoculated into 600 ml broth, kept in the light at 34.5°. After 49 h, portion transferred aseptically to another flask and kept at room temperature. Full circles and squares: culture at 34.5°; open circles and squares: portion placed at room temp.; full lines and circles: TPN enzyme; full lines and squares: DPN enzyme; broken lines and circles: chlorophyll; broken lines and squares: cells.

cells are returned to room temperature, a rapid formation of chloroplasts takes place after a lag period of 1 day[12]. Again the production of the TPN enzyme parallels the increase in chlorophyll. The DPN enzyme, on the other hand, behaves like a normal cellular constituent, as evidenced by a comparison of the curves for cellular multiplication and DPN enzyme production.

Distribution of the TPN GPDase in the cell fractions

Most of the TPN GPDase activity was found in the soluble fraction (see Table III). The possibility that the enzyme was originally present in the chloroplasts, and had leaked out during the fractionation procedure, must, however, be considered. The disruption of the cells lasted 3 min and the subsequent centrifugation of the chloro-

plasts 20 min. The enzyme molecules could have diffused from the plastids into the soluble fraction during this 25-min period. Any subsequent enzyme diffusion should have led to activity in the "chloroplast-supernatant" fraction. The lack of activity in this fraction indicates that if any diffusion had occurred, it was completed within the initial 25-min period. Although the results tend to indicate that the enzyme is normally present in the soluble fraction of the cytoplasm, a loose association with the chloroplasts is also possible.

TABLE III

DISTRIBUTION OF TPN GPDASE IN CELL FRACTIONS OF *Euglena*

Fraction	*% of total activity*
Chloroplasts	9
Chloroplast-supernatant	2.5
Mitochondria and microsomes	1.5
Soluble fraction	87

DISCUSSION

The formation of chloroplasts in resting colorless *Euglena* cells exposed to light is accompanied by a redistribution of protein material and a high protein turnover. New protein molecules are doubtless synthesized during this process, and the TPN-linked GPDase represents one of the enzymes connected with photosynthesis that are formed along with the chloroplasts. The site of synthesis of this enzyme is not known. In spinach leaves, a substantial fraction of the total enzymic activity can be found in the chloroplasts[4,13], and the considerable activity in the soluble fraction is thought to result from diffusion during the preparation. In the green *Euglena* cells, only a small portion of the total activity is found in the isolated plastids, but a rapid diffusion into the cytoplasm during the manipulations cannot be excluded.

Exposure of the colorless cells to light brings about the appearance of the TPN enzyme, and removal of the light stimulus results in the cessation of enzyme formation. This behavior is similar to that of many induced enzymes in bacteria. In this case, however, induction by light initiates a complex chain of events, and it is doubtful that light is the primary inducer of the TPN-linked GPDase. Since one of the effects of light during photosynthesis is the production of reduced TPN[14], it is tempting to speculate that this substance acts as an inducer for the formation of the TPN enzyme. This would require that functional photosynthesic units are formed soon after exposure of the colorless cells to light, since the production of the enzyme begins very early in the greening process.

The cessation of enzyme production when light is removed can be explained reasonably by assuming that photosynthetic activity is necessary for its synthesis. In the case of the bleaching treatment, however, chloroplast formation is inhibited in the presence of light. Photosynthesis presumably occurs under these conditions, and the bleaching temperature itself has very little effect on the production of the enzyme (Table II). Therefore one should not necessarily expect a strict relation between amount of enzyme formed and number of chloroplasts produced during the bleaching treatment. Yet the increases in TPN enzyme and chloroplasts appear

strictly linked under these conditions. Also after return of the culture to room temperature, both processes show the same lag period followed by an identical rapid rise.

The close relation between the production of TPN-linked GPDase and the formation of chloroplasts during partial bleaching followed by recovery suggests that it is the quantity of plastid structure, rather than the activity of the plastids, that determines the extent of enzyme synthesis. An understanding of the manner in which such a regulation could operate must await more definite evidence on the localisation of the enzyme in the cells, and possibly some knowledge of the site of TPN–GPDase synthesis. If the enzyme were produced in the plastids and remained associated with their structure, a direct control of the amount of enzyme in each plastid could be visualized. The problem of regulation should, however, be much more intricate if the localization and the site of synthesis of the enzyme were outside the chloroplast structure.

ACKNOWLEDGEMENTS

We are grateful to Dr. E. CHARGAFF for his interest and encouragement, and to Miss C. REBMAN for technical assistance. This work was supported by research grants from the American Cancer Society, the National Science Foundation and the United States Public Health Service.

REFERENCES

1 D. I. ARNON, *Science*, 116 (1952) 635.
2 M. GIBBS, *Nature*, 170 (1952) 164.
3 D. I. ARNON, L. L. ROSENBERG AND F. R. WHATLEY, *Nature*, 173 (1954) 1132.
4 L. L. ROSENBERG AND D. I. ARNON, *J. Biol. Chem.*, 217 (1955) 361.
5 R. H. HAGEMAN AND D. I. ARNON, *Arch. Biochem. Biophys.*, 57 (1955) 421.
6 G. BRAWERMAN AND E. CHARGAFF, *Biochim. Biophys. Acta*, 31 (1959) 164.
7 G. BRAWERMAN AND E. CHARGAFF, *Biochim. Biophys. Acta*, 31 (1959) 172.
8 R. C. FULLER AND M. GIBBS, *Plant Physiol.*, 34 (1959) 324.
9 E. G. KREBS, G. W. RAFTER AND J. M. JUNGE, *J. Biol. Chem.*, 200 (1953) 479.
10 C. F. CRAMPTON, R. LIPSHITZ AND E. CHARGAFF, *J. Biol. Chem.*, 206 (1954) 499.
11 M. GIBBS, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. I, Academic Press, New York, N.Y., 1955, p. 411.
12 G. BRAWERMAN AND E. CHARGAFF, *Biochim. Biophys. Acta*, 31 (1959) 178.
13 F. R. WHATLEY, M. B. ALLEN, L. L. ROSENBERG, J. B. CAPINDALE AND D. I. ARNON, *Biochim. Biophys. Acta*, 20 (1956) 462.
14 A. V. TREBST, H. Y. TSUJIMOTO AND D. I. ARNON, *Nature*, 182 (1958) 351.