ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 78, 407–415 (1958)

Yeast Uridine Diphosphogalactose-4-epimerase, Correlation between Activity and Fluorescence

Elizabeth S. Maxwell, Huguette de Robichon-Szulmajster and Herman M. Kalckar

From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, United States Department of Health, Education, and Welfare, Bethesda, Maryland

Received May 27, 1958

Introduction

Yeast UDP-galactose-4-epimerase[1] [formerly called galactowaldenase (1)] is an adaptive enzyme which catalyzes a reversible interconversion of UDP-glucose into UDP-galactose (1).

The properties of this enzyme differ in many ways from that of the mammalian epimerase (2–4). The mammalian epimerase requires small amounts of DPN and is inhibited by minute amounts of reduced DPN (3). Correspondingly, DPNase from extracts of *Neurospora* or hemolyzates of human erythrocytes eliminates the activation of epimerase by DPN (4).[2]

Yeast epimerase, in contrast, is highly active without the presence of free DPN. Moreover, reduced DPN is not inhibitory to this enzyme nor is DPNase. In the present article, the properties of a blue fluorescent enzyme obtained from galactose-induced yeast and its correlation with UDPGal-4-epimerase will be described.

Experimental

Materials

DPN, DPNH, UDPG, and PCMB were purchased from Sigma Chemical Company; UDPGal was prepared enzymically from UDPG and Gal-1-P as previously described (3); DEAE cellulose was obtained from Eastman Kodak Company.

[1] The following abbreviations have been used: DPN and DPNH for oxidized and reduced diphosphopyridine nucleotide, respectively; UDPG for uridine diphosphoglucose; UDPGal for uridine diphosphogalactose; PCMB for p-chloromercuribenzoate; DEAE-cellulose for N,N-diethylaminoethylcellulose; DPNase for diphosphopyridine nucleotidase.

[2] Also, H. N. Kirkman and H. M. Kalckar, unpublished.

Analytical Methods

Spectrophotometric measurements were made with a Beckman model DU spectrophotometer adapted by means of a pinhole filter for use with 0.5 ml. volume in a 1.0-cm. silica cell.

Fuorometric measurements were made in an Aminco-Bowman spectrophotofluorometer (5).

Protein was determined by the method of Lowry *et al.* (6). The possible release of DPN from purified yeast 4-epimerase was checked for in a slightly aged preparation by adsorption and elution from Darco KB. The eluate was tested for DPN by its stimulatory effect on purified calf liver 4-epimerase (3).

Preparation of UDPGal-4-epimerase from Yeast

Crude Extract. Saccharomyces fragilis was grown in a yeast extract salt medium containing either 5% glucose or 5% galactose as the main carbon source. Cultures were incubated 24 hr. at room temperature, and the cells were harvested and washed with cold water in a Sharples centrifuge.

The wet cell masses were suspended in 0.1 *M* glycine at pH 6.5 (200 g. cells/500 ml. suspension), and the cells were broken by 30-sec. exposure in a Nossal disintegrator. Cell debris was removed by centrifugation at about 10,000 × *g*. The clear supernatants from 200 g. of glucose-grown cells and from 200 g. of galactose-grown cells were carried through the following purification procedure simultaneously.

Removal of Nucleic Acid and Ammonium Sulfate Fractionation. To each 100 ml. of extract, 5 ml. of 1 *M* $MnCl_2$ was added and the precipitate was centrifuged and discarded.

Solid ammonium sulfate was added to the supernatant to bring the concentration to 65% saturated (39.8 g./100 ml. extract). The precipitates were centrifuged and dissolved in 50 ml. of cold distilled water. The ammonium sulfate concentration of the solutions as determined on the Barnstead Purity meter, was about 15% saturated. Solid ammonium sulfate (9.6 g.) was added to bring the final concentration to 50% saturated. The precipitates were centrifuged, dissolved in 15 ml. of cold distilled water, and dialyzed for 5 hr. against running distilled water. The precipitates which formed during dialysis were centrifuged and discarded. The clear supernatant from the glucose-grown cells contained about 110 mg. and that from the galactose-grown cells about 240 mg. protein.

DEAE Cellulose Column Chromatography. The entire sample from the glucose-grown cells and half that from the galactose-grown cells were adsorbed on two separate columns, each containing 5 g. DEAE cellulose (7, 8) washed with 0.01 *M* sodium phosphate buffer pH 7.1. The columns were developed simultaneously by a gradient-elution method using a single reservoir and mixing flask. The mixing flask contained 500 ml. of 0.01 *M* phosphate buffer pH 7.1 and the reservoir 500 ml. of 1 *M* NaCl in 0.01 *M* phosphate buffer. Fractions were collected at 3-min. intervals, and the rate of flow was approximately 1.3 ml./min. Each fraction was examined for UDPGal-4-epimerase activity, protein, and fluorescence. During the purification of the enzyme, UDPGal-4-epimerase activity was measured spectrophotometrically as previously described (7). UDPGal was used as the substrate,

and an excess of UDPG dehydrogenase (9) and DPN as the indicator system. A unit of activity was defined as an increase in optical density at 340 mμ of 0.001/min. under the condition of the assay. In cases where the effect of added DPN on 4-epimerase activity was studied, it was necessary to preincubate UDPGal and the enzyme fraction under investigation in the presence or absence of DPN. After stopping the reaction by boiling, UDPG formed during the preincubation was measured by adding DPN and UDPG dehydrogenase.

Results

Relationship of Fluorescence and Enzymic Activity

Marked differences were repeatedly observed in the ammonium sulfate fractions prepared from glucose- and galactose-grown cells. Only traces of both UDPGal-4-epimerase activity and fluorescent material were found in the preparations from glucose-grown cells, while those from galactose-grown cells contained from 4000 to 30,000 units of epimerase activity/mg. protein and exhibited a readily detectable fluorescence when examined in the Aminco-Bowman spectrophotofluorometer. The fluorescence spectrum resembled that of DPNH, having an activation maximum at 350 mμ and an emission maximum at 450 mμ. Figure 1 shows a copy of the recordings of fluorescence in the 0–50% ammonium sulfate fractions from both glucose- and galactose-grown cells.

Fractions collected in the DEAE cellulose column chromatography likewise showed a correlation between fluorescence and UDPGal-4-epimerase activity. Figure 2 shows the relationship of UDPGal-4-epimerase activity and fluorescence in each fraction collected from the preparation from galactose-grown yeast. Similar fractions collected from the preparation from glucose-grown cells contained only traces of the enzymic activity and displayed barely detectable fluorescence. The pattern of elution of protein for both preparations is shown in Fig. 3.

Effect of PCMB

Velick (10) and Racker and Krimsky (11) have shown that protein-bound DPN in crystalline muscle triosephosphate dehydrogenase can be removed by *p*-chloromercuribenzoate. Concomitant with PCMB treatment, the characteristic absorption band of the enzyme–DPN complex disappears. As illustrated in the present article, the fluorescence in the fractions from galactose-grown cells decreased with time after the addition of PCMB. This is illustrated in Fig. 4. The fluorescence

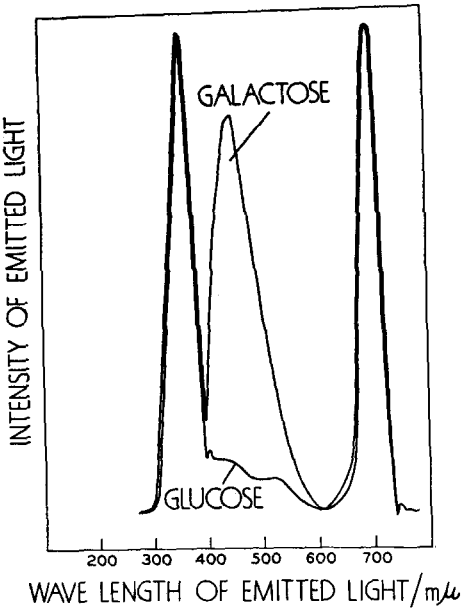

FIG. 1. Fluorescence in 0–50% ammonium sulfate fractions of extracts of galactose- and glucose-grown cells. Activation wavelength, 350 mμ; sensitivity, 0.03. The peaks at 350 and 700 mμ are due to scattered light. The peak at 450 mμ is fluorescence.

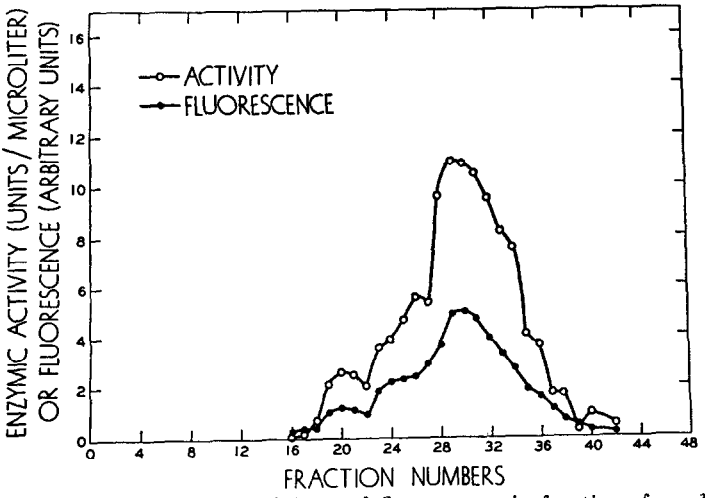

FIG. 2. UDPGal-4-epimerase activity and fluorescence in fractions from DEAE cellulose chromatography of galactose-grown cell preparations.

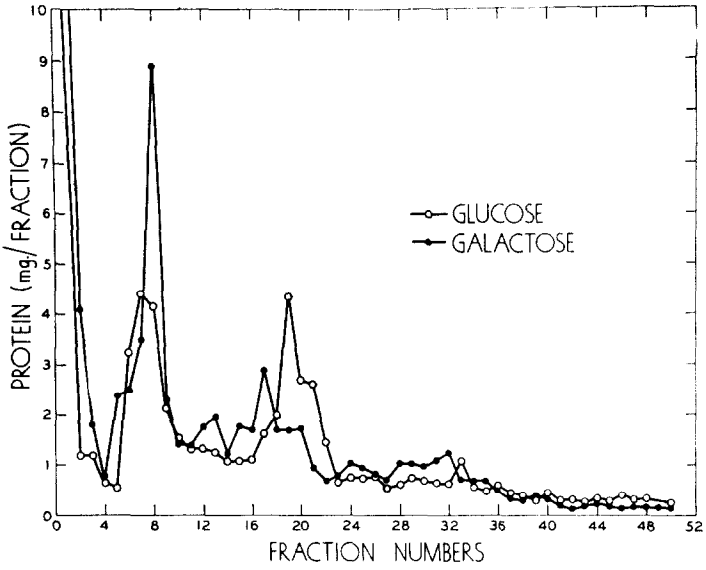

FIG. 3. Pattern of elution of protein in glucose- and galactose-grown yeast cell preparations from DEAE cellulose column.

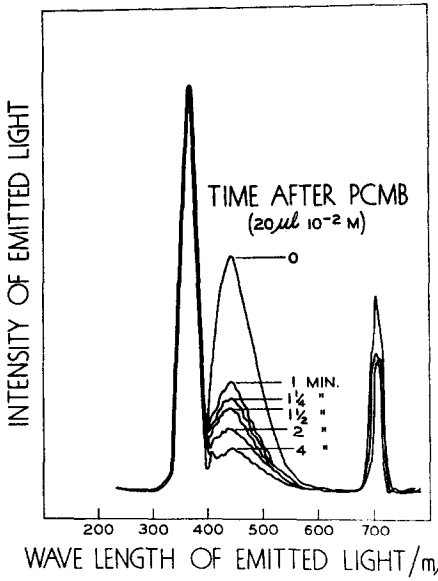

FIG. 4. Effect of PCMB on fluorescence in fraction 30 from DEAE cellulose chromatography of galactose-grown cell preparations. (Activation wavelength, 350 mμ; sensitivity, 0.01). Peaks at 350 and 700 mμ are due to scattered light. The peak at 450 mμ is fluorescence.

TABLE I

Effect of DPN on PCMB-Treated Enzyme

One milliliter of each fraction was treated with 10 μl. PCMB, 10^{-2} *M*, for 10–30 min. Protein was precipitated with ammonium sulfate and redissolved in 1 ml. water. Incubation mixtures contained 10 μl. enzyme, 43 mμmoles UDPGal, 10 μmoles cysteine, and 60 mμmoles DPN (where indicated) in a total volume of 0.5 ml. of 0.1 *M* glycine pH 8.7. Incubation was at room temperature for 30 min. UDPG formed was determined with UDPG dehydrogenase.

Fraction number	UDPG formed	
	With DPN	Without DPN
	mμmoles	*mμmoles*
Pooled 28 and 29	16.6	2
30	8.4	2

could not be restored by the addition of cysteine and DPN. However, when the PCMB-treated protein was precipitated with ammonium sulfate and redissolved in water, it could readily be shown that DPN was now required for UDPGal-4-epimerase activity. No DPN effect was found in freshly collected preparations untreated with PCMB. This is illustrated in Table I.

Fractions from the column chromatography which had been stored frozen for several weeks showed a decrease in both fluorescence and activity to about ⅓ of the original values. This is illustrated in Table II. In these fractions UDPGal-4-epimerase activity was stimulated 15–40% by the addition of DPN to the assay system. A release of DPN under these circumstances is under investigation.

Discussion

The fluorescent spectrum of yeast UDPGal-4-epimerase is reminiscent of that of reduced DPN, both with respect to maximum of the fluores-

TABLE II

Effect of Storage[a] *on Fluorescence and Enzymic Activity*

Fraction number	Fluorescence		Activity[b]	
	Units	Per cent original	Units/ml.	Per cent original
32	10	20	1.9	20
33	10	30	2.6	32
34	9	33	2.2	29

[a] Samples were stored frozen for 5 weeks with occasional thawing and refreezing.

[b] Assayed in presence of DPN and UDPG dehydrogenase.

cence spectrum as well as to the efficiency of the exciting light as a function of wavelength (12).

Protein-bound reduced DPN has shown in some cases a fluorescent spectrum with a maximum at 415 mμ (13); in some cases the maximum is closer to that of free DPNH (14, 15).

For various reasons we want to pose the question whether protein-bound unreduced DPN may not show fluorescence. Our suspicion that yeast 4-epimerase may contain bound DPN is prompted by the following facts:

1. The mammalian UDPGal-4-epimerase needs DPN (4); the activity brought about by the latter is abolished by the addition of DPNase (4) and suppressed by addition of DPNH (3). The crude or freshly purified yeast 4-epimerase does not need DPN and is neither inhibited by DPNase nor by DPNH. One plausible interpretation is that it operates in a manner analogous to the liver epimerase but has the DPN "built in" (cf. the fluorescent cyanide-DPN complex (16)).

2. Treatment of the yeast enzyme with PCMB brings about a resolution, i.e., the fluorescence as well as the activity disappear. Addition of DPN and cysteine restores activity but not fluorescence.

3. On one occasion with purified yeast epimerase which had aged moderately at −10°, it has been possible to detect small amounts of free DPN as assayed by the highly sensitive and specific calf liver 4-epimerase test. One or 2 weeks later, DPN could not be detected any more. Corresponding tests on fractions from glucose-grown yeast did not release any DPN. This aspect is under current investigation.

4. The fact that PCMB treatment of yeast 4-epimerase converts it to an enzyme which requires DPN for its activity indicates that the active DPN complex which may also involve UDP hexose is somehow dependent on sulfhydryl groups on the enzyme. Such a structure may give rise to fluorescence. It is also possible that a "ternary" complex including protein (SH groups), DPN, and UDP-glucose or galactose may permit the existence of "hydride cycle" in which part of the time the DPN is in the reduced form.

A determination of the number of DPN molecules per molecule enzyme protein is greatly warranted, as is a determination of essential sulfhydryl groups in the protein. It would also be worth trying to look for enzyme-bound UDPG or UDPGal. Obviously, further purification of the enzyme is needed for such purposes.

The fact that neither tritiated DPN nor tritiated reduced DPN (chemically prepared) donate tritium to UDPG or UDPGal in detectable

amounts, if incubated with a highly active liver 4-epimerase (3), is still difficult to interpret. It may mean that free reduced DPN does not mix with a transitory free reduced DPN formed in a ternary complex. It is also possible that the transitory DPNH formed is reduced on a carbon of the pyridine ring different from that in the para position. A different alternative would be a mechanism in which reduced DPN is not formed at all. There are at least two possibilities: (*a*) A transfer of a hydrogen atom creates a free radical which does not proceed to the formation of DPNH. The free radical is part of a ternary complex. (*b*) A proton (rather than a hydride ion or hydrogen atom), originating from the carbon 4 of the hexose, might be shared by the participants of the ternary complex during the 4-epimerization process. Studies with tritium-labeled pyridine nucleotides as applied to the yeast 4-epimerase are under way.

The problem of biological interconversion of galactose to glucose may in some way or another be closely related with that of racemization, a field to which Dr. H. O. L. Fischer, to whom this article is dedicated, has contributed so much.

Acknowledgment

The authors wish to express appreciation to Mr. Randolph Frye for valuable technical assistance in the preparation of the enzymes.

Summary

Yeast grown on galactose contains a strikingly fluorescent protein in the fractions which contain UDP-galactose-4-epimerase. UDP-galactose-4-epimerase from yeast, unlike the corresponding enzyme from liver, does not require addition of diphosphopyridine nucleotide for activity. Further purification for the yeast enzyme gives a close correlation between enzyme activity and fluorescence. Addition of *p*-chloromercuribenzoate brings about a disappearance of fluorescence as well as activity. The latter can be restored by addition of diphosphopyridine nucleotide. A discussion of possible mechanisms of the enzymic interconversion poses a number of new problems.

References

1. Leloir, L. F., *Arch Biochem. Biophys.* **33,** 186 (1951).
2. Maxwell, E. S., Kalckar, H. M., and Burton, M. R., *Biochim. et Biophys. Acta* **18,** 444 (1955).

3. MAXWELL, E. S., *J. Biol. Chem.* **229,** 139 (1957).
4. MAXWELL, E. S., *J. Am. Chem. Soc.* **78,** 1074 (1956).
5. BOWMAN, R. L., CAULFIELD, P. A., AND UDENFRIEND, S., *Science* **122,** 32 (1955).
6. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J., *J. Biol. Chem.* **193,** 265 (1951).
7. PETERSON, E. S., AND SOBER, H. A., *J. Am. Chem. Soc.* **78,** 751 (1956).
8. SOBER, H. A., GUTTER, F. J., WYCKOFF, M. M., AND PETERSON, E. A., *J. Am. Chem. Soc.* **78,** 756 (1956).
9. STROMINGER, J. L., MAXWELL, E. S., AXELROD, J., AND KALCKAR, H. M., *J. Biol. Chem.* **224,** 79 (1957).
10. VELICK, S. F., *J. Biol. Chem.* **203,** 563 (1953).
11. RACKER, E., AND KRIMSKY, I., *J. Biol. Chem.* **198,** 731 (1952).
12. DUGGAN, D. E., BOWMAN, R. L., BRODIE, B. B., AND UDENFRIEND, S., *Arch. Biochem. Biophys.* **68,** 1 (1957).
13. BOYER, P. D., AND THEORELL, H., *Acta. Chem. Scand.* **10,** 477 (1956).
14. THEORELL, H., AND BONNICHSEN, R., *Acta Chem. Scand.* **5,** 1106 (1951).
15. DRYSENS, L. N. M., AND KRONENBERG, G. H. M., *Biochim. et Biophys. Acta* **26,** 437 (1957).
16. COLOWICK, S. P., KAPLAN, N. O., AND CIOTTI, M. M., *J. Biol. Chem.* **191,** 447 (1951).