ENHANCED FLUORESCENCE OF 4-EPIMERASE ELICITED BY 5'-URIDINE NUCLEOTIDES+

A. Bhaduri,* A. Christensen, and H. M. Kalckar

Biochemical Research Laboratory,
Harvard Medical School,
and Massachusetts General Hospital,
Boston, Massachusetts 02114

Received November 22, 1965

As mentioned in the preceding article (Creveling et al., 1965), UDP-galactose 4-epimerase isolated from induced Saccharomyces fragilis shows a characteristic blue fluorescence of varying intensity. It has been found recently (Bhaduri et al., 1965) that this fluorescence can be greatly increased by incubating the native fluorescent enzymes with a number of 5'-uridine nucleotides. The activity of UDP-glucose [Sigma] persists after 40 minutes hydrolysis at 100° and pH 2; this effect can be replaced by UDP + glucose. However, 5'-uridylate + glucose (or galactose) is the most efficient fluorescence promoter encountered so far.

Fluorescence of epimerase incubated with 5' -uridylate (10^{-3}M)

+ Supported by grants from the National Institutes of Health (AM 05507 and AM 05639) and from the National Science Foundation (GB-3646), by the Joseph P. Kennedy, Jr. Foundation and by the Wellcome Trust. We are grateful to Dr. R. A. Darrow for many valuable discussions.

* Present address: 43G Ultadingi Road, Calcutta 4, India.

and glucose (10^{-2} M) doubles within 30 minutes and quadruples within 2 hours. If a more moderately fluorescent epimerase preparation is used (Creveling et al., 1965) fluorescence increases of about 20 fold are frequently seen. Marked responses can be obtained with concentrations as low as 5×10^{-4} M 5'-uridylate, the glucose concentration being 10^{-3} M. Using such concentrations, 10 to 20 fold increases of fluorescence ensue within 4 to 5 hours at 25° (the so-called UMP/G factor; see Fig. 1).

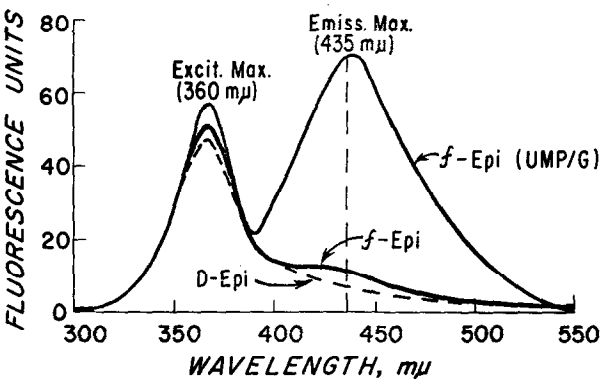

Fig. 1. Formation of a highly fluorescent reduced DPN complex by addition of 5'-uridylate 10^{-3} M and glucose 10^{-2} M to f-epimerase (1.2 mg per ml in 0.1 M Tris buffer, pH 7.4). The strongly fluorescent epimerase is called "f-Epi (UMP/G)". D-epimerase, 1.2 mg per ml in the same buffer, Incubation for 5 hours at 25°. Excitation at 360 mμ (see Creveling et al., 1965). Emission maximum at 435 mμ.

We shall call the epimerase fluorescence factor formed from UDP + glucose "UDP/G", whereas that formed from 5'-UMP + glucose will be called "UMP/G". The maximally fluorescent epimerase generated by either UDP/G or UMP/G will be called $\mathfrak{F}_1$ in analogy with the terminology described previously (Creveling et al., 1965).

We shall summarize our terminology for all the various states of the epimerase proteins as follows: pCMB-protein, non-fluorescent, inactive epimerase-protein obtained by reacting all the 15 SH groups of the diomer with pCMB (Creveling et al., 1965); D-epimerase, dark epimerase, reactivated non-fluorescent epimerase which requires DPN addition for catalytic activity, obtained from pCMB-protein after removal of the mercurial by

mercaptoethanol; f-epimerase, native epimerase with a fluorescence less than 20% of $\mathfrak{F}_H$ (Creveling et al., 1965); F-epimerase, native epimerase with a fluorescence higher than 20% of $\mathfrak{F}_H$ (Creveling et al., 1965); $\mathfrak{F}_H$-epimerase, native epimerase maximally reduced by BH_4 (Creveling et al., 1965); $\mathfrak{F}_U$-epimerase, native epimerase with maximal fluorescence due to UMP/G or UDP/G.

$\mathfrak{F}_H$-epimerase and $\mathfrak{F}_U$-epimerase resemble each other as follows:

(i) Increase in absorption at 340 mμ; only 30% of the increase of $O.D._{340}$ can be accounted for as 1.4 DPNH (cf. Creveling et al., 1965).

(ii) Decrease of catalytic activity accompanying increase in fluorescence and $O.D._{340}$. $\mathfrak{F}_U$ as well as $\mathfrak{F}_H$ show only 5% of original native activity.

(iii) Decrease of DPN. $\mathfrak{F}_U$ and $\mathfrak{F}_H$ have retained less than 10% of the original DPN.

(iv) Emission maxima at 435 mμ. Fluorescence enhancement amounts to 25 to 30 fold.

(v) Quantum yield of fluorescence of generated reduced DPN very high, order of magnitude of 80 to 90%.

$\mathfrak{F}_U$ and $\mathfrak{F}_H$ differ, however, in these respects:

(a) The fluorescence increase up to the $\mathfrak{F}_U$ level is not rapid, as in the case of BH_4 reduction, forming $\mathfrak{F}_H$, but relatively slow. The maximum fluorescence (10 to 25 fold that of the native fluorescence) is reached within 6 to 10 hours at room temperature. (The additional fluorescence, "the $\mathfrak{F}_U$ fluorescence", is largely retained after precipitation with 70% saturated ammonium sulfate, washing with saturated ammonium sulfate and redissolution in Tris buffer.)

(b) UDP + glucose as well as 5' uridylate + glucose are able to elicit fluorescence of D-epimerase. Such an effect has not been observed for BH_4.

The formation of fluorescent UMP/G (or UDP/G) complexes of D-epimerase are already conspicuous after 1 hour. The effect requires small amounts of DPN; this is apparent from the fact that fractionation of D-epimerase with ammonium sulfate (see next section) prevents restoration of fluorescence by uridylate and glucose until DPN is again supplemented.

DPN and reduced DPN in various forms of epimerase.

If an f-epimerase is precipitated with ammonium sulfate, spun, and redissolved in Tris buffer, the bound DPN remains with the protein and can be determined directly by the Lowry method (Lowry, Roberts and Kapphann, 1954). Such a preparation contains approximately 1 mμmole DPN per mμmole protein (based on dimer units of approximately 120,000 M. W. (Darrow and Rodstrom, 1966). However, the same preparation treated with pCMB (15 mμeq. per mμmole) prior to ammonium sulfate precipitation retains only 0.07 mμmole DPN in the ammonium sulfate precipitate. Hence more than 90% of the bound DPN has been lost. In contrast, the reduced DPN present in native F-epimerase does not seem to be released from D-epimerase, although its enhanced fluorescence disappears (Creveling et al., 1965)

The loss of DPN from D-epimerase may explain why this nucleotide is required for the fluorescence effect of uridylate and glucose as well as for the restoration of catalytic activity.

The type of reduced DPN of the UMP/G or UDP/G fluorescence factors is only partly known. As in the case of borohydride reduction, 30% of the reduced bound DPN can be accounted for as 1.4 DPNH after treatment with pCMB which brings about a marked decrease of fluorescence.

$\mathfrak{F}_v$-epimerase contains reduced DPN and has lost most of its

TABLE I

Changes in Bound Pyridine Nucleotide and Enzyme Fluorescence Induced by 5'-Uridylic Acid and Glucose.

	mμmole bound ("Epi"*) or free pyridine nucleotide			ε340	Fluor. U.** x 10^{-3} at 435 mμ pr mμmole enz. ++	Fluor. Enhancm. $\frac{\text{Epi DPN}^{red.}}{\text{free 1.4 DPNH}}$
	Epi $DPN^{red.}$ (pr mg enz.)	Epi DPN (pr mg enz.)	ΔEpi DPN (pr mg enz.)			
f-epim.+	non-detect.	10			3.0	no determ.
f-epim.+ (UMP/G)	8.8	1	9	6,000	67.5	27.0

\+ 1.5 mg f-epi/ml incubated with and without 5'-uridylate + glucose.

* Epi $DPN^{red.}$ -- Epimerase bound reduce DPN. ϵ_{340} for Epi $DPN^{red.}$ assigned 6,200.

Epi DPN -- Epimerase bound DPN (Lowry et al., 1957). The ϵ_{340} for the Epi $DPN^{red.}$ based on DPN consumption is app. 6,000 (see column 5).

** Fluorescence units as related to the fluorescence of a quinine standard of 2.2 μg quinine per ml 0.1 NH_2 SO_4.

++ Based on a M. W. of 120,000 (Darrow and Rodstrom, 1966).

DPN and more than 90% of its catalytic activity. The formation of reduced DPN and the loss of DPN can be determined quantitatively. Based on these observations, the extinction coefficient at 340 mμ of the reduced DPN generated in $\mathfrak{F}_H$ amounts to approximately 6,000 (see Table I).

The enhancement of fluorescence is between 25 and 30 and hence

TABLE II

Quantum Yields of Fluorescence of Various Reduced Forms of DPN.

Various forms of reduced DPN	Fluorescence[+] per μmole DPNH/ml	Emiss. max. mμ	Mol. ϵ_{340}	Quantum yield %
1.4 DPNH (free)	2.5	450	6,250	3
F-epim.	app. 65	435	not determ.	app. 90
f-epim. reduced by BH_4	65.2	435	5,900[++]	app. 90
f-epim. +UMP/G	64.5	435	6,000[++]	app. 88

\+ Fluorescence in "Quinine units", based on the fluorescence of a quinine reference (see Table I) Excitation maximum in all cases 360 mμ

* Quantum yields related to Weber's determination of the absolute quantum yield of the fluorescence (Weber, 1958).

++ ϵ_{340} for the bound reduced DPN generated was determined as described on the basis of the DPN consumption brought about by UMP + glucose (see Table I of this article). The ϵ_{340} for free DPNH was assessed to 6,200.

substantially larger than any fluorescence enhancement of related types hitherto desceibed (Yonetani and Theorell, 1962). On the basis of Weber's determination of the absolute quantum yield of 1.4 DPNH (Weber, 1958) and with estimates of fluorescence as well as extinction coefficients of various types of reduced bound DPN, it is possible to compare quantum yields of fluorescence. It can be seen that the types of reduced bound DPN generated in $\mathfrak{F}_H$ as well as in $\mathfrak{F}_0$ have a quantum yield of fluorescence as high as 90% (see Table II).

F-epimerase contains about 0.6 to 0.7 mμmole DPN and about 0.3 to 0.4 mμmole of reduced DPN per mμmole. Since the native blue fluorescence has an excitation maximum identical to that of free DPNH, it is presumably due to a form of bound reduced DPN. Based on the DPNH and DPN determinations mentioned above, the quantum yield of the native fluorescence (with an emission maximum of 435 mμ) is likewise of the order of 85 to 90%.

Addition of specific substrate (UDP-glucose or UDP-galactose in amounts approximately 0.2 to 1 μmole per ml) brings about a reduction of enzyme-bound DPN (Wilson and Hogness, 1964) albeit without any detectable increase in fluorescence. Apparently the reduced DPN generated by the specific substrate is strongly quenched. This is an exceptional feature which presumably is due to another conformational change of the epimerase brought about specifically by substrate.

REFERENCES

Bhaduri, A., Darrow, R. A., Kalckar, H. M., and Randerath, E. (1965): Fed. Proc. 24, 1932.

Creveling, C. R., Bhaduri, A., Christensen, A. Darrow, R. A., and Kalckar, H. M. (1965): See preceding pages this issue.

Lowry, O. H., Roberts, N. R., and Kapphann, J. I. (1957): J. Biol. Chem. 224, 1047.

Darrow, R. A . and Rodstrom, R. E. (1966): Proc. Nat. Acad. Sci., to be communicated by H. M. Kalckar.

Weber, G. (1958): J. Chem. Physics 55, 878.

Yonetani, T. and Theorell, H. (1962): Arch. Biochem. Biophys. 99, 433.

Wilson, D. B. and Hogness, D. S. (1964): J. Biol. Chem. 239, 2469.