MOLECULAR TRANSITIONS IN AN INDUCED ENZYME WITH ENHANCED FLUORESCENCE[+]

C. R. Creveling, * A. Bhaduri, ** A. Christensen, and H. M. Kalckar

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

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UDP-galactose 4-epimerase isolated from Saccharomyces fragilis induced by galactose exhibits a blue fluorescence having an excitation maximum of around 350 mμ and an emission maximum of 435 mμ (Maxwell et al., 1958; Maxwell and de Robichon-Szulmajster, 1960). It contains various bound nucleotides. UDP-glucose and UDP-galactose have been identified by enzymatic methods (Creveling et al., 1964a). The other types of nucleotides, identified in some preparations chemically as well as enzymatically, are DPN and 1.4 DPNH (Maxwell et al., 1958; Creveling

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* Present Address: National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda 14, Maryland.

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

et al., 1964a; Creveling et al., 1964b). Quantitative determinations of DPN and DPNH together with sedimentation studies by Darrow and Rodstrom (1966) show the following stoichiometrical relations. Each unit of 6S or approximately 120,000 M.W. contains only one molecule of pyridine nucleotide, either DPN or reduced DPN, in spite of the fact, that, according to Darrow and Rodstrom (1966), such units of 120,000 are composed of two smaller subunits of approximately 60,000 M.W. Furthermore, although most of the fluorescence of native epimerase, if not all, may be attributed either to bound reduced DPN or to related DPN complexes having an absorption maximum at 340 mμ, the fraction of the total DPN which is present in the reduced form is always less than one-third, even in the most strongly fluorescent preparations of native epimerase (see below).

We have confirmed the previous observations (Maxwell et al., 1958) that during purification the fraction with the highest specific catalytic activity always shows blue fluorescence. It should be stressed, however, that the fluorescence per unit of catalytic activity in the most purified epimerase can vary, depending upon the treatment of the yeast. Highly fluorescence epimerase preparations can be obtained from yeast grown on galactose and exposed to galactose during and after autolysis. Such strongly fluorescent purified epimerases show a distinct absorption maximum at 260 mμ. (O.D. at 260 is 1.7 times that of 280, which indicates the presence of additional nucleotides.) In preparations from yeast which has been washed free of galactose and freeze-dried prior to autolysis, one usually finds fluorescence to be less pronounced. These more "dim" fluorescent epimerases show the usual absorption maximum for proteins at 280 mμ.

Preparations of various forms of epimerase.

It is possible by means of borohydride in the presence of a small amount of substrate to convert practically all the enzyme-bound DPN to DPNH under conditions which permit strong fluorescence (Darrow et al., 1963). We shall call this completely "reduced" enzyme $\mathfrak{F}_H$-epimerase and relate in a semi-quantitative way the fluorescence of the various native enzymes to the $\mathfrak{F}_H$-epimerase. Let the maximum fluorescence level, $\mathfrak{F}_H$, arbitrarily represent the 100% level. Those native fluorescent epimerases which show fluorescence below 20% of maximum will be called f-epimerases, whereas those showing fluorescence above 20% of maximum (usually 30 to 50% of $\mathfrak{F}_H$) will be called F-epimerases.

The ratio between DPN and DPNH in the f-epimerase is probably close to 10 or higher. In any case, DPNH could not be detected. In F-epimerase, the ratio DPN/DPNH has been determined and found to range between 3 and 5. Hence even a strongly fluorescent native enzyme always contains more DPN than DPNH; the DPNH was identified as 1.4 DPNH (Creveling et al., 1964b).

If the sulphydryl groups of epimerase are permitted to react with p-chloromercuric benzoate (pCMB) the fluorescent preparations become non-fluorescent (Maxwell et al., 1958). Fluorescent epimerase, (whether a strongly fluorescent reduced enzyme, for instance $\mathfrak{F}_H$-epimerase, or a "dim" f-epimerase) consumes 120 mμeq. of pCMB per mg protein, and at this end point fluorescence has vanished (see Fig. 1). Catalytic activity vanishes after the consumption of about half of the available SH groups. This is also illustrated in Fig. 1 which represents a titration of an F-epimerase. Each mμmole epimerase "dimer" contains 14 to 15 mμ equivalents of SH groups, assuming a molecular weight of 120,000 for the 6S particle.

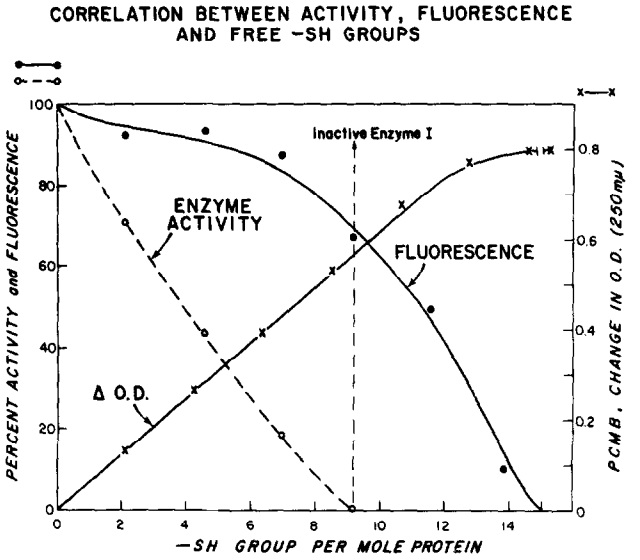

Fig. 1. Purified F-epimerase (pretreated with Sephadex G-25) was dissolved in Tris buffer pH 7.4; concentration of epimerase was 1 mg per ml, and was assayed for epimerase activity according to the method of Darrow and Creveling (1964) and expressed in per cent of the activity of untreated purified F-epimerase. The sulphydryl groups were titrated with p-chloromercuric benzoate (pCMB) according to the method of Boyer et al., (1954). Fluorescence emission at 435 mμ (maximum) was followed in microquartz cuvettes with crossed prisms in the Bowman-Aminco spectrophotofluorometer. Excitation maximum in this instrument was found to be 360 mμ for free as well as bound DPNH. The per cent fluorescence relates to the fluorescence of purified untreated F-epimerase, at the emission maximum of 435 mμ.

Enzyme which has been completely titrated with pCMB has a sedimentation coefficient of 4S (Darrow and Rodstrom, 1966), is non-fluorescent, catalytically inactive, and not responsive to reactivation with DPN unless the mercurial is removed by sulphydryl reagents (Maxwell et al., 1958). The dark epimerase thus obtained is called "D-epimerase". For catalytic activity, D-epimerase requires the addition of DPN (Maxwell et al., 1958) since it has lost its bound DPN. The reduced DPN of F-epimerase remains bound even in the dark epimerase albeit in a quenched state (quantum yield equal or lower than that of free 1.4 DPNH).

Since borohydride is able to reduce the bound DPN and thus greatly increase the native fluorescence of the epimerase (See Fig. 2), we have tried to determine quantitatively the enhancement factor of fluorescence of reduced DPN generated in the $\mathfrak{F}_H$ form of epimerase. As can be seen from Table I, the enhancement, if based on the fluorescence of free 1.4 DPNH, amounts to a factor of 25 to 30, which is more than 5 times larger than fluorescent enhancement hitherto described (Yonetani and Theorell, 1962).

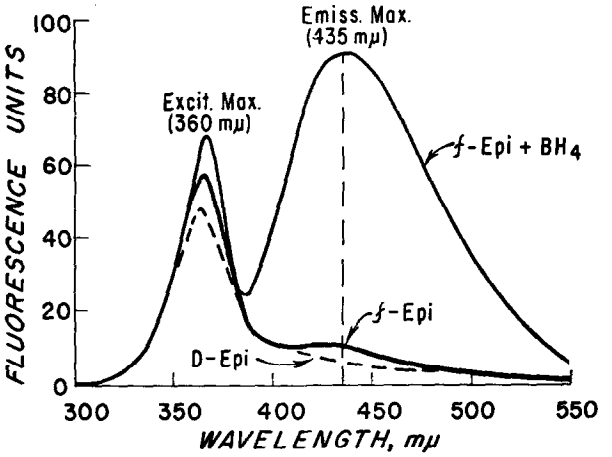

Fig. 2. Reduction of epimerase DPN by BH_4. UDP-galactose is present to stabilize borohydride effect (see text). For further detailed information, see legend to Fig. 1 and footnotes to Table I.

The presence of a small amount of substrate (50 mμmoles of UDP-glucose or UDP-galactose per mg protein) tends to stabilize the borohydride reduced epimerase and to promote complete reduction, although the small amounts of substrate by themselves do not give rise to any detectable increase in fluorescence

The type of reduced DPN formed in $\mathfrak{F}_H$ -epimerase has been only partly characterized. If the $\mathfrak{F}$ -epimerase is converted to D-epimerase the bound reduced DPN is partly accessible to enzymatic reoxidation. Addition of lactic dehydrogenase plus an excess of pyruvate reoxidizes about 30% of the reduced DPN generated by borohydride.

TABLE I

Fluorescence Enhancement of Reduced Epimerase-bound DPN as Generated by BH_4.

	mμmole bound ("Epi") pyridine nucleotide*		Fluor. U** x 10^{-3} at 435 mμ per mμmole enz. ++	Fluor. enhancm. Epi $DPN^{red.}$ / free 1.4 DPNH
	Epi $DPN^{red.}$ (pr mg enz.)	Epi DPN (pr mg enz.)		
f-epim.	non-detect.	8.3	3.0	
f-epim. +BH_4*	7.3+	0.7	66.0	27

* To 1.57 mg purified f-epimerase in 1 ml o.1 M Tris buffer, pH 7.4, was added 0.05 μmole UDP-galactose and 0.05 ml of a 0.2 M glycine buffer, pH 11, containing 150 μg BH_4. Bound reduced DPN (Epi $DPN^{red.}$) was determined by changes in optical density between 300 and 400 mμ (assigning an ϵ_{340} of the Epi $DPN^{red.}$ of 6,200). The optical changes were normalized against the same amount of enzyme, UDP-galactose and 0.05 ml 1 M glycine containing borate in an amount corresponding on a molar basis to that of the BH_4 present in the experimental cuvette. Epimerase DPN, called Epi DPN, was determined by the method of Lowry et al., (1957). Fluorescence excitation maximum at 360 mμ (see legend to Fig. 1); fluorescence emission maximum at 435 mμ.

\+ Of this 2.1 mμmole could be accounted for as 1.4 DPNH, using lactic acid dehydrogenase and pyruvate.

** Fluorescence units as related to the fluorescence of a quinine standard of 2.2 μg quinine per ml 0.1 N H_2SO_4.

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

It should be noted that in contrast to the native F-epimerase, the F_N-epimerase has lost more than 90% of its catalytic activity. However, at a pH around 8, it retains activity because of the instability of the BH_4 reduction at alkaline reaction (Darrow et al., 1963). This instability manifests itself by a decrease of fluorescence down to the original native level. At the same time, catalytic activity is almost completely restored.

Hence the epimerase protein remains essentially intact during reduction with BH_4. However, the reduction of practically all the bound DPN is not compatible with full catalytic activity. It should be emphasized that variations in the levels of native fluorescence although often 3 to 5 fold are not reflected in the catalytic activity. The restoration of fluorescence in D-epimerase will be discussed in a subsequent article (Bhaduri et al., 1965b)

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