ON THE DIVERSITY OF -SH GROUPS IN DPNH DEHYDROGENASE AND THEIR TENTATIVE LOCALIZATION*

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Received August 19, 1966

It has been known for many years (Mahler and Elowe, 1954) that DPNH-cytochrome c reductase preparations from heart mitochondria are rapidly and completely inhibited by -SH inhibitors, such as p-chloromercuribenzoate (PCMB). The inhibition is competitive with respect to cytochrome c (Minakami et al., 1963) and has been observed in all cytochrome-reducing derivatives of mitochondrial DPNH dehydrogenase examined (Watari et al., 1963). This rapidly reacting -SH group is not manifest in the soluble or particulate preparations of the dehydrogenase prior to modification of the protein and will be referred to as type I.

Relatively intact forms of the dehydrogenase, as present in purified, soluble preparations or in ETP and ETP_H particles, are rather insensitive to inhibition by mercurials in the DPNH-ferricyanide assay, particularly in the cold and in phosphate buffer (Singer and Ringler, 1961; Minakami et al., 1963, 1964). As shown by Cremona and Kearney (1965), however, when the catalytically active PCMB, PCMS, or N-ethylmaleimide (NEM) derivatives of the enzyme, prepared and freed from unreacted inhibitor at 0°, are incubated at temperatures between 15° and 30° (where the untreated enzyme is stable), loss of activity in the ferricyanide assay develops gradually at a rate and to an extent governed by the temperature. Thiols do not reverse this type of inactivation, although they prevent its development if the enzyme-mercaptide is dissociated prior to raising the temperature. The -SH groups involved in the initial combination with PCMB, PCMS, or NEM will be referred to as type II in

* Supported by grants from the National Heart Institute (HE 10027), the National Science Foundation (GB 3762), the San Francisco Heart Association, and by Contract No. 5024(00) between the Office of Naval Research and the University of California.

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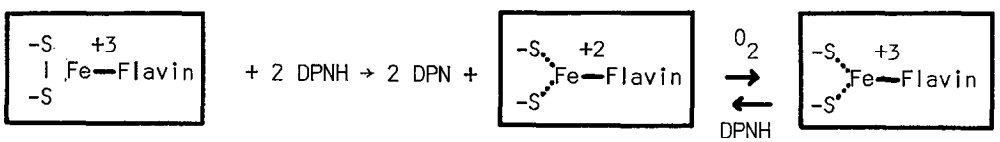
this paper. It is thought that when these groups are tied up in covalent linkage with the inhibitor, the protein is labilized and that the inactivation observed is an expression of a conformation change. The modification appears to involve the nonheme iron region of the protein, since only assays involving iron-labile sulfide prosthetic groups are affected (ferricyanide reduction, EPR signal at g=1.94) while the substrate-flavin interaction is unaffected, since full transhydrogenase activity is retained (Cremona and Kearney, 1965). Type I and II -SH groups are distinct since after type II groups are reacted with NEM, the enzyme may be degraded to a fully active DPNH-cytochrome c reductase in which type I groups are free.

Recently Tyler et al. (1965 a, b) reported the existence of still another type of -SH group (Type III) in the enzyme. They found that brief preincubation of ETP with DPNH predisposes the respiratory chain-linked DPNH dehydrogenase to rapid and extensive inhibition on subsequent addition of mercurials. They postulated that the sulfur groups involved are initially present in the disulfide state which is reduced by DPNH to an iron-flavin semi-quinone chelate, which is oxidized by O_2 via the respiratory chain to a third form, the one capable of rapid reaction with mercurials:



The postulated role of type III thiol in DPNH oxidation and, hence, its location near the substrate binding site rests on spectrophotometric observations on the state of the flavin. Since spectral data do not yield reliable information on the redox state of the flavin with this enzyme (Minakami et al., 1963), it was desirable to obtain evidence that the inhibition affects the DPNH-FMN interaction. Another purpose of this study was to ascertain the relation, if any, of type III -SH groups to types I and II.

Table I illustrates the behavior of type III -SH groups in the DPNH - O_2 reaction in ETP preparations. Comparison with the data of Tyler et al. confirms the marked effect of pretreatment with substrate on the inhibition produced by mercurials, but in our hands mercurials always produce a significant inhibition even without prior incubation ("preconditioning") with DPNH. This is not unexpected, since at room temperature type II -SH groups will also react gradually. (Cremona and Kearney, 1965).

Table I. Effect of pretreatment with DPNH on the sensitivity of DPNH dehydrogenase to mersalyl in ETP preparations.

Condition of Pretreatment	Specific activity* Data of Tyler et al. (1965)	Data from this lab.
A. None (control)	1.32	1.26
B. Mersalyl, then DPNH	1.05	0.67
C. DPNH, then mersalyl, then DPNH	0.12	0.16
D. Same as C, but $Fe(CN)_6^{-3}$ before mersalyl	1.0	0.77

*Polarographic O_2 uptake at room temperature; μmoles DPNH/min./mg. Experimental conditions were as per Tyler et al. (1965a). Mersalyl, where present, 15 μM; DPNH during pretreatment 0.2 mM; during assay 0.4 mM. In B reaction was started with DPNH after 1 minute incubation with mersalyl; in C after the initial amount of DPNH was exhausted, mersalyl was added and incubated 1 minute, then more DPNH was added. In D after DPNH was exhausted, 50 μM $Fe(CN)_6^{-3}$ was added; then after 1 minute mersalyl, then DPNH were added.

In order to ascertain that type III groups are indeed localized in the dehydrogenase, similar experiments were conducted with the purified soluble enzyme as well as with ETP and ETP_H both at room temperature and at 0°, where binding of type II groups does not result in inactivation. Inactivation of the DPNH-ferricyanide reaction gave a qualitatively similar pattern to that shown in Table I. Hence, the type III groups are clearly localized in the primary dehydrogenase. Since the ferricyanide assay, which involves nonheme iron, and the transhydrogenase assay, which represents a direct reaction of the substrate with enzyme-bound FMN, are inhibited to comparable extents (Table 2), it appears to be verified that type III groups are located near the substrate or flavin sites. The participation of the S groups in the catalytic cycle, as postulated by Tyler et al. (1965 a, b), may not follow from available data for several reasons, however.

First, at 0 to 2°, where both the reduction of the enzyme by DPNH and its reoxidation by the cytochrome chain take less than 1 msec., mercurial inhibition, after "preconditioning" with DPNH (condition C in the Tables), is slow and incomplete. Inactivation in the cold seems to follow a biphasic curve: there is a relatively fast initial phase followed by an extremely slow secondary one. In a typical experiment after 30 min. at 1.8° inactiva-

tion reached only 50%. This behavior suggests that the action of DPNH during the preconditioning phase is not merely a reduction of -SS- groups but more probably the initiation of a series of conformation changes in the protein.

Table 2. Effect of pretreatment with DPNH on the sensitivity of DPNH dehydrogenase to mersalyl in ETP preparations.

Condition of Pretreatment	Inactivation (%) In $Fe(CN)_6^{-3}$ assay	Inactivation (%) In transhydrogenase assay
A. Control	0	0
B. 1×10^{-4} M mersalyl for 5 min., then DPNH	14	20
C. DPNH for 3 min., then mersalyl for 5 min.	75	77
D. DPNH for 8 min., no mersalyl	21	15

Ferricyanide and DPNH-acetylpyridine DPN transhydrogenase assays, both at V_{max} as per Minakami et al. (1963). Mersalyl, where present, 0.1 mM; DPNH during pretreatment 0.4 mM. Treatments were at 20°, assays at 30°.

Second, loss of ferricyanide activity of the purified dehydrogenase in condition C is always accompanied by the emergence of cytochrome c reductase activity, which also suggests a conformation change. Third, the "preconditioning" with DPNH itself regularly produces a loss of activity (e.g., Table 2, condition D). This is in line with the finding of Rossi et al. (1965) that prolonged contact between the dehydrogenase and reduced substrates leads to extensive conformational changes and eventually to fragmentation. Conceivably, "preconditioning" of the enzyme by DPNH to inhibition by mercurials and NEM may be merely a symptom or stage of the conformational alterations set in course on incubation of the protein with DPNH.

The following observations distinguish type II and III -SH groups.
(1) Combination of the former with mercurial or NEM near 0° does not result in loss of catalytic activity; combination of the latter with these reagents leads to progressive inactivation, although, as noted above, the kinetics are complex in the cold.
(2) Combination of the former with negatively charged -SH inhibitors (PCMB, PCMS) is prevented by inorganic phosphate and, to a lesser extent, by DPNH,

while inactivation by attack on type III groups requires DPNH and proceeds much better in phosphate than in Tris or triethanolamine buffers, in accord with the data of Tyler et al. (1965 a, b).

(3) The loss of activity initiated by binding of type II groups results in loss of ferricyanide but not of transhydrogenase activity, while that due to type III groups is reflected in both assays.

(4) The former type of inactivation is not accompanied by the appearance of cytochrome c reductase activity; the latter type is.

(5) Ferricyanide abolishes the "preconditioning" effect of DPNH toward type III groups but does not affect the reactivity of type II.

(6) Lastly, the two types of groups may be reacted with mercurials one at a time. This was shown by preparing the fully active PCMS derivative at 0° and removing unreacted PCMS as per Cremona and Kearney (1965). The derivative was completely stable in the cold but was rapidly inactivated at 30°, in accord with the observations of Cremona and Kearney. An aliquot of the derivative, which had been kept cold, was then treated for 10 minutes with 9×10^{-5} M DPNH at 0° and upon subsequent addition of 2×10^{-4} M mersalyl 70% inactivation developed in 60 minutes.

Prior studies by Cremona and Kearney have clearly shown the non-identity of type I and II -SH groups. The fact that type I and III are distinct was shown by "preconditioning" the purified enzyme with DPNH then inactivating it with 2×10^{-4} M mersalyl (1 hr. at 0°), and then removing excess mercurial on Sephadex G-25. The resulting preparation, along with an untreated control, was then converted to cytochrome reductase by 4 hours digestion with B. subtilis proteinase. The yield of DPNH cytochrome c reductase was the same in both samples and the -SH groups required for cytochrome reduction (which emerge during proteolysis) were in the free state, since the cytochrome reductase activity thus created could be readily inhibited by mercurials.

It appears from these and previous studies that DPNH dehydrogenase in unmodified form probably does not contain any -SH groups which are directly involved in catalysis but contains structural sulfhydryls. Three distinct types of -SH groups may be demonstrated under suitable conditions. Type II may be present as such in the native enzyme; types I and III may be present bu unreactive and become reactive toward mercurials as a result of conformation changes or may arise as a result of structural modification. The possible existence of a fourth type of -SH is suggested by studies in Estabrook's laboratory (Minakami et al., 1964). It is reported to play a role in electron flux from DPNH to CoQ or O_2 but not to ferricyanide in a respiratory chain preparation. At this time data are insufficient to permit its localization in the dehydrogenase or comparison with the three types discussed above.

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