EVIDENCE FOR THE EXISTENCE AND FUNCTION OF AN OCCULT, HIGHLY REACTIVE SULPHYDRYL GROUP IN THE RESPIRATORY CHAIN DPNH DEHYDROGENASE *

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Studies by Minikami et al (1) of the influence of mercurials on the DPNH oxidation system of heart muscle particles, the ETP preparation (2), have revealed the existence of a slowly reacting sulphydryl group which is essential for DPNH oxidase activity, but not essential for either DPNH ferricyanide reductase activity or the reduction of flavin by DPNH. Recently, we have found that when heart muscle particles are subjected to a brief phase of DPNH oxidation, they now contain a rapidly reactive mercurial-sensitive component. The rapidly reacting site, in contrast to the slowly reacting site (1), is essential for DPNH-ferricyanide reductase activity, the reduction of flavin by DPNH, and the formation of the DPNH-linked EPR signal (g = 1.94) component of the respiratory chain linked DPNH dehydrogenase.

RESULTS AND DISCUSSION

A comparison of the oxygen electrode traces A and B, presented in Fig. 1, shows the remarkable increase in the sensitivity of the DPNH oxidation

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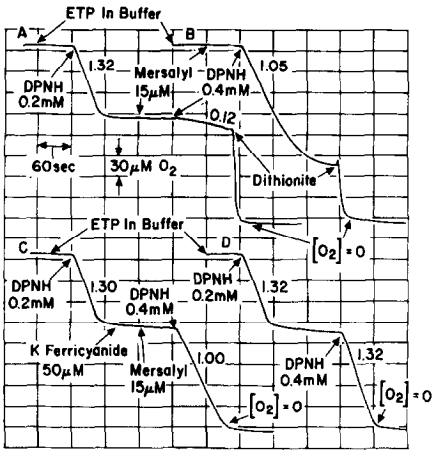

Fig. 1. Influence of mersalyl on the DPNH oxidase activity of heart muscle particles. Oxygen uptake was assayed polarographically with a Clark oxygen electrode (3). In each experiment, the reaction mixture initially contained ETP (0.6 mg. protein) in 3.0 ml. of air saturated 50 mM K phosphate buffer, pH 7.4. Numbers against the traces express DPNH oxidase activity as μmoles DPNH consumed/minute/mg. protein. Temperature, 22° C.

system to the mercurial, sodium mersalyl, which occurred after the pretreatment or 'preconditioning' of the heart muscle particles with DPNH. In trace A, incubation of the DPNH-pretreated system for one minute with 15 μMolar mersalyl resulted in more than 90 % inhibition of DPNH oxidase activity, whereas in experiment B, which used non pretreated particles, the degree of inhibition was only about 20 %. Similar results were obtained when 15 μMolar concentrations of either p-chloromercuribenzoate or p-chloromercuriphenyl sulphonate were used instead of mersalyl. No enhanced sensitivity to mercurials was observed when the particles were pretreated with 0.2 mMolar DPN instead of DPNH. The pretreatment phenomenon was observed in phosphate, triethanolamine or bicarbonate buffers, and showed no specific anion or cation requirement. Studies on the kinetics of inhibition of DPNH oxidase activity showed that the DPNH pretreatment increased the reactivity of the particles with mercurials by about 60-fold.

The experiment of Fig. 1, trace C, shows that the addition of ferricyanide to the DPNH pretreated particles abolished their rapid react-

ivity with mersalyl.The addition of mMolar levels of imidazole or ADP in place of ferricyanide had a similar influence,but ferrocyanide or uncoupling agents were without effect.

The DPNH-ferricyanide reductase activities of particles inhibited by mersalyl at the rapidly reacting site (with DPNH pretreated ETP) and the slowly reacting site (with non pretreated ETP),were compared under conditions in which the DPNH oxidase activity of both systems was strongly inhibited by the mercurial.It was found (Table I) that mersalyl inhibition of DPNH oxidase activity at the slowly reacting site did not affect DPNH-ferricyanide reductase activity,in agreement with the previous report (1),whereas inhibition at the rapidly reacting site also resulted in a strong inhibition of the DPNH-ferricyanide reductase reaction.

In experiments reported elsewhere (4),it has been found that

Table 1. Influence of mersalyl on DPNH oxidase and DPNH-ferricyanide reductase activities

Preparation	DPNH Oxidase activity*	DPNH-$Fe(CN)_6$ activity*	% Inhibition Oxidase	% Inhibition Reductase
DPNH pretreated ETP	1.28	2.7	-	-
" + mersalyl	0.09	0.4	93	85
Non pretreated ETP	1.30	3.0	-	-
" + mersalyl	0.03	3.0	98	Nil

Oxidase and reductase activities were measured as described elsewhere (1). Pretreated ETP was prepared by allowing the particles to completely oxidize 0.2 mMolar DPNH before the addition of mersalyl. The ETP was incubated with 15 μMolar mersalyl for 30 mins. (pretreated ETP) and 60 mins. (non pretreated ETP) before assay. * μmoles DPNH consumed/minute/mg. protein.

the reduction of flavin by DPNH was abolished by mersalyl inhibition at the rapidly reacting site,but was not prevented by inhibition at the slowly reacting site.In view of these findings,it was of interest to establish whether the EPR g = 1.94 iron signal,which is induced by treatment of heart muscle

particles with DPNH (ref. 5 and Fig. 2, trace A), was observed in particles inhibited by mercurials at the slowly and the rapidly reacting sites. The EPR iron signal associated with the DPNH dehydrogenase flavoprotein is most suitably measured by treating the particles with rotenone before the addition of DPNH, since in the absence of rotenone, DPNH also induces most of the g = 1.94 iron signal associated with the succinate dehydrogenase flavoprotein (4). The influence of mersalyl on the DPNH dehydrogenase EPR signal component is shown in the EPR spectra of Fig. 2. When the particles were inhibited by mersalyl at the slowly reacting site, a DPNH-linked g = 1.94 signal similar to that observed in the rotenone and DPNH treated particles

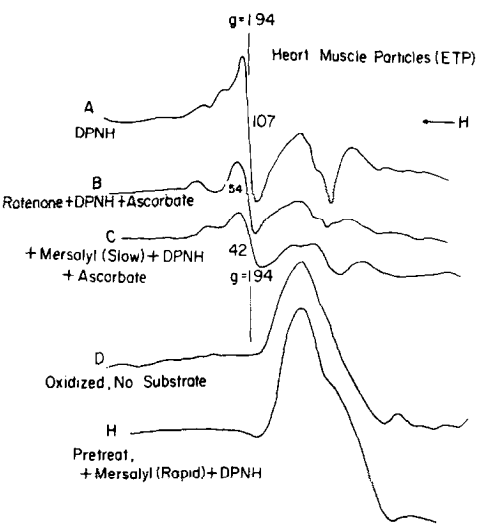

Fig. 2. EPR spectra (first derivative) of heart muscle particles. Each sample, treated with substrates and inhibitors at 23°C, consisted of a 0.5 ml. suspension of ETP (25 mg. protein) in 0.1 Molar phosphate buffer, pH 7.4. Concentrations of other reagents used were:- DPNH, 6 mMolar; rotenone, 3 mMolar; ascorbate, 20 mMolar; and mersalyl, 5 mMolar. In experiment E, the particles were pretreated in dilute suspension with 0.2 mMolar DPNH and 15 μMolar mersalyl, sedimented by centrifugation, resuspended and treated with DPNH. Spectra were taken at liquid nitrogen temperature in a Varian Model V4280 A EPR Spectrometer. Numbers against the traces express the peak to peak amplitude of the g = 1.94 signal, in arbitrary units.

was obtained (traces B and C). Both preparations were treated with ascorbate to prevent the appearance of the large copper signal of oxidized cytochrome oxidase (trace D), which might otherwise have interfered with the g = 1.94

signal.When the rapidly reacting site was blocked with mersalyl before treatment with DPNH,no iron signal was observed and only the copper signal of cytochrome oxidase was present in the system.

The studies summarized above suggest that functional sulphydryl groups are situated in the DPNH oxidation system of heart muscle particles at the positions indicated in Fig. 3.Inhibition with mercurial at

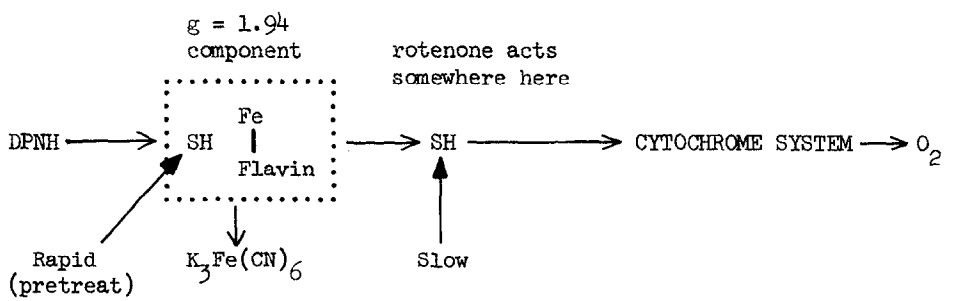

Fig. 3. Postulated relationships between sulphydryl groups,the rotenone-sensitive site and the substrate induced EPR iron signal (g = 1.94) component of the DPNH oxidation system of heart muscle particles.

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the rapidly reacting site leads to loss of DPNH oxidase activity,DPNH ferricyanide reductase activity,and also the loss of appearance of the EPR iron signal upon treatment with DPNH.In contrast,inhibition at the slowly reacting site leads only to the loss of DPNH oxidase activity,without affecting the other DPNH-linked reactions.

The results obtained in this study also show that three enzymatic states of heart muscle particles can be distinguished.Two of the states (DPNH pretreated and non pretreated) differ markedly in their sensitivity to mercurials,but neither state is found to exhibit an EPR iron signal.In the third enzymatic state,the particles do show an EPR g = 1.94 iron signal,due to treatment with DPNH in the absence of electron transport (in the rotenone inhibited state or anaerobic state).The influence of DPNH,oxygen,mercurials and ferricyanide on the interconversion of these three enzymatic states is shown in the reaction scheme of Fig. 4.

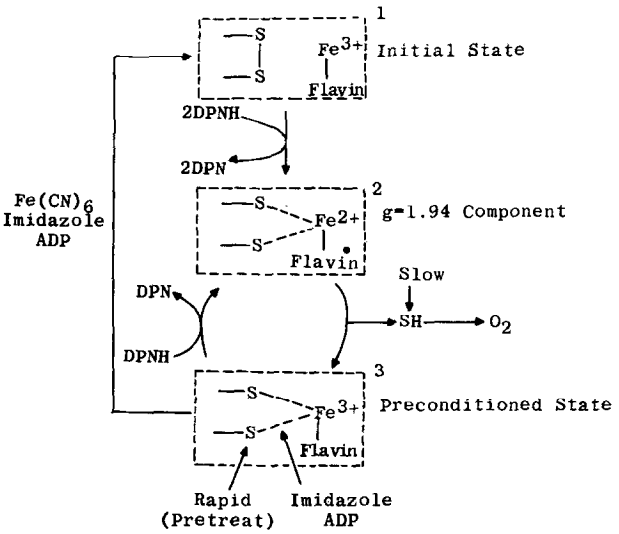

Fig. 4. A reaction scheme showing the participation of sulphydryl groups, non heme iron and flavin in the oxidation of DPNH by the respiratory chain linked DPNH dehydrogenase.

According to the scheme, treatment of ETP in the initial state (containing a mercurial-insensitive disulphide group) with two moles of DPNH per mole of enzyme flavin, leads to the appearance of the full EPR g = 1.94 iron signal (6). The signal is postulated to be due to the formation of an iron-flavin semiquinone complex, containing sulphur-iron ligands. In the presence of oxygen, the complex is converted to an oxidized form, present in the preconditioned state, and containing mercurial-sensitive sulphur-iron ligands. The oxidation of DPNH is shown to be mediated by the shuttling of electrons between the semiquinoid and the oxidized forms of the flavin moiety and the concomitant oxidation-reduction of the non heme iron complexed with the flavin. The relationships between imidazole, ADP and the iron-flavin complex of the DPNH dehydrogenase flavoprotein, and their possible significance in the coupling mechanism at the DPNH-flavin region of the respiratory chain, have been discussed recently elsewhere (7).

In summary, our studies support the hypothesis that a hitherto undetected sulphydryl grouping is essential for the activity of

the respiratory chain linked DPNH dehydrogenase, and is intimately associated with a non heme iron flavin complex.

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