Preliminary Notes

PN 10029

A flavoprotein oxidizing NADPH isolated from liver microsomes

In previous work[1,2] evidence was presented that hepatic microsomes possess a peculiar NADPH oxidase requiring vitamin K_3 (2-methyl-1,4-NQ), 1,4-NQ or 1,2-NQ as cofactor. This paper describes briefly the purification of this oxidase and reports its possible identity with a microsomal flavoprotein, NADPH-cytochrome *c* reductase (EC 1.6.2.3), which has been isolated by previous workers[3-5]. A mechanism is also presented for the NQ-dependent oxidation of NADPH by this flavoprotein.

Rabbit-liver microsomes prepared by a modification[6] of the method of MITOMA *et al.*[7] were digested at 0° for 15 h with 0.07% crude pancreatic lipase ("steapsin") in 0.1 M phosphate buffer (pH 7.4). The digest was centrifuged at 105 000 × *g* for 60 min and the supernatant was fractionated with ammonium sulphate. A low activity of a NQ-independent NADPH oxidase[1,2,8] was thereby precipitated between 0 and 0.50 satn., whereas the precipitate obtained between 0.50 and 0.75 satn. contained most of the NQ-requiring oxidase. The latter fraction was dialysed and treated with calcium phosphate gel in 0.01 M phosphate buffer (pH 6.0). After washing the gel with water, the NQ-requiring oxidase was eluted from the gel with 0.2 M potassium phosphate buffer (pH 7.0). The eluate was dialysed against 5 mM phosphate buffer (pH 6.7), and applied to a hydroxylapatite column equilibriated with the same buffer. On washing the column with phosphate buffer (pH 6.7) of increasing concentrations, the oxidase started to migrate as a pale-yellow band and was eluted out at the phosphate concentration of 0.07 M.

The preparation thus obtained represented about a 200-fold purification over microsomes and catalysed, in the presence of 20 μM vitamin K_3, the oxidation of NADPH at a rate of 10–15 μmoles/min/mg protein with concomitant consumption of oxygen (20°, 0.33 M phosphate buffer, pH 6.5). The activity was dependent on the ionic strength of reaction medium. NADH was less than 1% as effective as NADPH as substrate. Ultracentrifugally, the preparation was nearly homogeneous, but was still contaminated by a small amount of impurities. The main component, with which the yellow colour was associated, has a sedimentation coefficient ($s_{20,w}$) of $4.55 \cdot 10^{-13}$ sec^{-1}. As shown in Fig. 1, the absorption spectrum of purified enzyme was characteristic of a flavoprotein with maxima at 275, 375–380 and 455 mμ. The band at 455 mμ was greatly diminished on addition of NADPH. The flavin was identified as FAD by the D-amino acid oxidase (EC 1.4.3.3) assay after liberating from the protein by heating at 100° for 5 min.

In addition to the NQ-dependent oxidation of NADPH by oxygen, the purified enzyme catalysed the NADPH-linked reduction of a variety of acceptors including, 1,4-NQ, 2-methyl-1,4-NQ 1,2-NQ, *p*-benzoquinone, 2,6-dichlorophenolindophenol neotetrazolium chloride and cytochrome *c*. Cytochrome b_5 and another microsomal

Abbreviations: NQ, naphthoquinone; NQH_2, naphthoquinol; NQH·, naphthosemiquinone.

cytochrome, P-420[8,9], which has recently been separated from cytochrome b_5 (ref. 10), were, however, not reduced. The reduction of the naphthoquinones to quinols was observable only under anaerobic conditions; aerobically they acted as cofactors for the aerobic oxidation of NADPH rather than being reduced. *p*-Benzoquinone, on the other hand, was reduced to hydroquinone even in the presence of oxygen.

This enzyme differs from DT diaphorase studied and so named by ERNSTER *et al.*[11,12] in that it is insensitive to dicoumarol and does not oxidize NADH. On the other hand, it is very similar in a number of respects to a hepatic NADPH-cytochrome *c* reductase previously purified by several workers[3-5]. It is highly probable that the two enzymes are identical with each other.

As mentioned above, this enzyme catalyses under anaerobic conditions the NADPH-linked reduction of naphthoquinones to the quinols at pH 6.5. The purified enzyme, however, did not accelerate the negligibly slow autoxidation of the quinols at pH 6.5. This lack of naphthoquinol oxidase activity in the purified enzyme excludes the possibility that quinols are involved in the NQ-mediated oxidation of NADPH. This was further supported by an experiment in which NADPH, 2-methyl-1,4-NQ and the enzyme were anaerobically incubated until all the vitamin added was reduced to the quinol form and then air was bubbled into the reaction system.

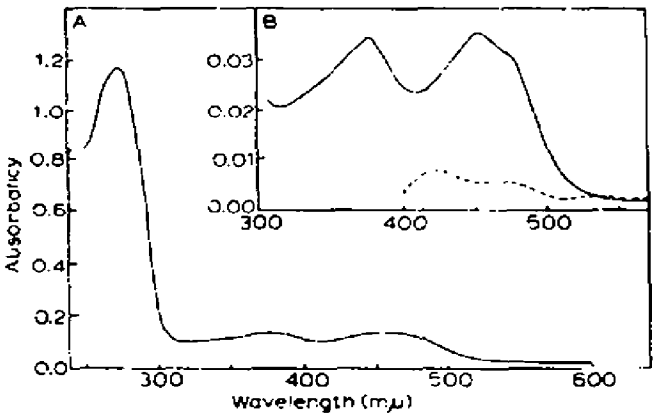

Fig. 1. Absorption spectra of a NQ-requiring NADPH oxidase purified from liver microsomes. (A) The oxidized form of a preparation having a specific oxidase activity of 5.7 μmoles NADPH oxidized/min/mg protein. Protein concentration, 0.55 mg/ml. 0.02 M Phosphate buffer (pH 6.7). (B) The oxidized (——) and NADPH-treated (- - - -) forms of a preparation having a specific activity of 13.3 μmoles NADPH oxidized/min/mg protein. Protein concentration, 0.185 mg/ml. 0.3 M Phosphate buffer (pH 6.5). NADPH (0.15 μmole) was added /ml of the oxidized enzyme solution.

No oxidation of NADPH was thereby observed immediately, and significant oxidation commenced only after a relatively long lag period. It is conceivable that the lag period is the time required for the regeneration of a sufficient amount of quinone, by the slow autoxidation of the quinol (at pH 6.5), to induce the active NADPH oxidation.

As has already been suggested[1], it is possible to explain this peculiar NADPH oxidase activity by assuming a mechanism involving a semiquinone rather than a quinol as the key reactant. The primary event in the overall process seems to be

the reduction of enzyme-bound flavin by NADPH, as suggested by the decrease in absorbancy at 455 mμ on addition of NADPH. Under anaerobic conditions, a NQ, also bound by the enzyme, will accept electrons from the reduced flavin to form NQH· and then NQH_2. If it is assumed that the NQH· is very labile to oxygen and rapidly reoxidizable to NQ by oxygen, probably because of its special linkage to the enzyme protein, it will be understandable that NQ mediates, in the presence of oxygen, the aerobic oxidation of NADPH without intermediate formation of NQH_2. The semiquinone form of *p*-benzoquinone may not be oxidizable by oxygen for reasons still to be investigated. The participation of a radical, monodehydroascorbate, has also been suggested in the ascorbate-stimulated oxidation of NADH by adrenal microsomes[13].

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The oxidation–reduction potential of cytochrome b_5 in soluble and particulate form with reference to the role of lipid

The oxidation–reduction potential is one of the important properties of respiratory enzymes. It generally determines the position of the enzymes in respiratory chains and contributes to understanding their function in the living cells. Concerning the potential of cytochrome b_5, a microsomal hemoprotein, several conflicting values have been reported. The potential determined first by YOSHIKAWA[1,2] with dog- and rabbit-liver preparations was shown to be −0.13 V. Later, STRITTMATTER AND BALL[3] obtained the same value with a rat-liver particulate suspension. The cytochrome from rabbit-liver microsomes was solubilized and purified by VELICK AND STRITTMATTER[4] who estimated its oxidation–reduction potential to be +0.02 V. In the Symposium on Hematin Enzymes held at Canberra[5], the cause of the discrepancy was subjected to debate, but no clear answer was obtained. The object of the present study is to clarify this question.