The Oxygen Requirement of an NADH Dehydrogenase from *Sepia officinalis*

During the course of recent work aimed at the identification of the NADH and NADPH dehydrogenases of the optic-lobes of *Sepia officinalis*[1,2], it was noticed that one of these enzymes, a dicumarol-sensitive NADH-ferricyanide reductase, capable also of oxidase activity, lost its diaphorase activity when assayed under anaerobic conditions.

The enzyme was partially purified[1] and represented the fraction salted out at 25% saturation of ammonium sulphate. Its activity was measured spectrophotometrically at 420 nm in cuvettes of 3 ml containing $10^{-4}M$ NADH ($5 \times 10^{-4}M$ potassium ferricyanide, $10^{-3}M$ GSH, $3 \times 10^{-3}M$ EDTA, and $0.05M$ potassium phosphate buffer pH 7.3).

The activity was also completely inhibited by pretreatment of the enzyme with oxygen-free argon or nitrogen for 5 min, but could be fully restored by bubbling oxygen (5 min) in the inactivated enzyme solution.

Cycles of inactivation and reactivation could be repeated more than once, thus indicating the reversible nature of this effect. The inability of the oxygen dissolved in the assay mixture to reverse the inactivation brought about by nitrogen (or argon) was traced to an inhibition by ferricyanide which produced a complete loss of activity when incubated with the active enzyme at the same concentration used in the assay (Table I). Ferricyanide prevented also the recovery of activity brought about by oxygen (Table II). On the other hand, NAD, NADH, NADP and NADPH were without appreciable effect on all these reactions. Similarly, 2,6-dichlorophenolindophenol, which was an active electron acceptor for the enzyme, was also without effect on these reactions. When added together with ferricyanide, NADH did not prevent its inhibitory effect.

Reduced glutathione and cysteine added to the enzyme solution before bubbling nitrogen prevented the loss of activity brought about by this treatment (Table I), and in addition, were also able to reactivate the N_2-inactivated enzyme (Table II). Oxidized glutathione, on the other hand, did not prevent nitrogen inactivation (Table I), was unable to produce any reactivation by itself, and completely inhibited oxygen-reactivation (Table II). Similar experiments could not be carried out meaningfully with cystine since this compound strongly inhibited the active enzyme (Table I). $FeCl_2$ partially prevented nitrogen inactivation (Table I) and even produced some reactivation of the nitrogen-treated enzyme (Table II). This observation was in accord with the inhibitory effect of *o*-phenanthroline on the active enzyme (Table I). The stimulatory effect of GSSG on the enzyme activity when added to the reaction mixture[1] may not be at variance with the effects observed upon incubation with the enzyme during nitrogen treatment and the enzyme during nitrogen treatment and oxygen reactivation.

It should be noted that in all the experiments described, the activity of the enzyme was assayed in presence of saturating concentrations of GSH and moreover that the activating effect of GSH and GSSG was found to be reversible upon dilution.

These data indicate that an interaction of oxygen with the enzyme is necessary for its activity and takes place presumably with a group in its reduced state, as shown by the contrasting effects of reduced and oxidized compounds; the effect of ferrous ion and the inhibition by *o*-phenanthroline may suggest the involvement of a metal. Such a possibility, however, requires further experimentation[3].

Table I. Effect of several compounds on the N_2 inactivation of the NADH dehydrogenase. Rates expressed in μmole/min

Compound	Control	N_2 treatment
None	0.37	0.00
GSH	0.45	0.37
GSSG	0.45	0.00
Cysteine	0.52	0.52
Cystine	0.00	0.00
Ferricyanide	0.00	0.00
o-Phenanthroline	0.00	0.00
$FeCl_2$	0.30	0.19

The effect of each compound ($3 \times 10^{-3}M$) on the enzyme activity was measured by incubating it with the enzyme solution in $0.05M$ potassium phosphate buffer pH 7.3. After 5 min 0.1 ml of the solution was withdrawn and added to 2.9 ml of the complete reaction mixture. The effect on the reaction of nitrogen inactivation was measured by adding the individual compounds at the same concentration to the enzyme solution before bubbling nitrogen for 5 min.

Table II. Effect of several compounds on the N_2-inactivated NADH dehydrogenase and on the O_2 reactivation of the inactive enzyme. Rates expressed in μmole/min

Compound	N_2-inactivated enzyme	O_2 treatment
None	0.00	0.30
GSH	0.30	0.27
GSSG	0.00	0.00
Cysteine	0.30	0.30
Cystine	0.00	0.00
Ferricyanide	0.00	0.00
o-Phenanthroline	0.00	0.00
$FeCl_2$	0.12	0.30

The effect of each compound on the reactivation by oxygen was measured by adding it to the solution of N_2-inactivated enzyme before bubbling O_2 for 5 min. The compound was considered to have produced a reactivation under similar conditions but without bubbling oxygen. Control experiments carried out by again bubbling nitrogen after addition of the compound ensured that the effect was not due to traces of oxygen introduced with the compound.

Riassunto. L'enzima dicumarolo-sensibile purificato da lobi ottici di *Sepia officinalis*, una NADH-deidrogenasi, con notevole attività ossidasica, è stato saggiato in condizioni anaerobiche. La presenza dell'ossigeno, come delle sostanze riducenti, in particolare dei gruppi SH, è essenziale per l'attività dell'enzima che privato di questi fattori si inattiva facilmente, mentre con la ripristinazione delle condizioni ottimali (ossigeno e sostanze riducenti incubate), si può ristabilire in gran parte l'attività enzimatica.

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[1] E. Aloj and A. Giuditta, J. Neurochem. *14*, 955 (1967).
[2] A. Giuditta and E. Aloj, J. Neurochem. *14*, 967 (1967).
[3] Thanks are due to Dr. A. Giuditta for suggesting this work and for much useful advice.