On the reaction mechanism of lipoyl dehydrogenase

It has been shown that lipoyl dehydrogenase from pig heart is a flavoprotein[1-3] identical with STRAUB[4] diaphorase. Further studies have shown that in catalysis, the flavin is functioning between the oxidized and semiquinoid levels[5], with only one interconversion of FAD and FADH$^{\cdot}$ per catalytic cycle. The fate of the other electron involved has been clarified by the finding that a second molecule of pyridine nucleotide and the conversion of a protein disulphide linkage to a protein dithiol are involved in the reaction. The reduction of lip S_2 by DPNH shows a lag period which occurs to varying extents with some commercial samples of DPNH but not with others and hence was attributed to the presence of an inhibitor[3]. Fig. 1 shows that this lag period

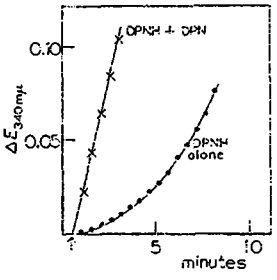

Fig. 1. The effect of DPN on the reduction of lip S_2 by DPNH. The reactions were carried out at 25° in a volume of 3 ml. Each cell (1 cm light path) contained 2500 μmoles citrate (pH 5.65), 3 μmoles EDTA, 2 mg serum albumin, 2 μmoles DL-lip S_2 and 0.6 μmole DPNH (freshly made Sigma 98%) $\pm$ 0.3 μmole DPN. Reaction was begun by addition of 1.4 μg pure diaphorase, and followed by the decrease in absorbancy at 340 mμ. The first reading was taken after 30 sec (indicated by arrow); all subsequent differences (corrected for blank rates in the absence of enzyme) were related to this reading.

TABLE I

SUBSTRATE-INDUCED DISULPHIDE–DITHIOL REACTION IN DIAPHORASE

In the experiments with diaphorase (0.35 mg/ml), enzyme was incubated at 0° with 0.03 M phosphate pH 6.3, $3 \cdot 10^{-3}$ M EDTA and the concentrations of reactants shown. Samples of 0.01–0.03 ml were withdrawn to test activity spectrophotometrically at 25°. In the lip S_2 assay each cell contained in a vol. of 3 ml, 2500 μmoles citrate pH 5.65, 3 μmoles EDTA, 2 mg bovine serum albumin, 2 μmoles DL-lip S_2, 0.3 μmole DPNH, 0.3 μmole DPN. Absorbancy changes were followed at 340 mμ after the addition of enzyme. The DCIP assays were performed as described previously[2]. In the experiments with KGD, enzyme (1.15 mg/ml) was incubated at 0° with 0.03 M phosphate pH 7.4, $3 \cdot 10^{-3}$ M EDTA and the concentrations of reactants shown. KGD was prepared as described previously[2]. Samples of 0.01–0.03 ml were taken for assays as described above.

	Relative activity	
	with LipS_2	*with DCIP*
Diaphorase at 0°	(100)	(100)
Diaphorase incubated with 10^{-3} M arsenite	100	100
Diaphorase incubated with $4 \cdot 10^{-4}$ M DPNH	100	1000
Diaphorase incubated with $4 \cdot 10^{-4}$ M DPNH + 10^{-3} M arsenite	17	1400
KGD complex at 0°	(100)	(100)
KGD complex incubated with 10^{-3} M arsenite	105	120
KGD complex incubated with $4 \cdot 10^{-5}$ M CoASH + $3 \cdot 10^{-3}$ M KG*	105	70
KGD complex incubated with $4 \cdot 10^{-5}$ M CoASH + $3 \cdot 10^{-3}$ M KG + 10^{-3} M arsenite*	8	70

* These reaction mixtures also contained 10^{-3} M cysteine.

Abbreviations: CoASH, coenzyme A; DPN, DPN$^{\cdot}$ and DPNH, oxidized, half-reduced and reduced diphosphopyridine nucleotide; EDTA, ethylenediaminetetraacetate; FAD, FADH$^{\cdot}$ and $FADH_2$, oxidized, semiquinoid and fully reduced flavin-adenine dinucleotide; KG, α-ketoglutarate; KGD, ketoglutarate dehydrogenase complex; lip S_2 and lip $(SH)_2$, oxidized and reduced lipoic acid; TPP, thiamin pyrophosphate.

is due to the production of DPN, which is required as a cofactor in the reaction. The function of a reactive disulphide group in the enzyme has been revealed by studies with arsenite, as shown in Table I. When diaphorase or KGD was incubated with 10^{-3} *M* arsenite and then diluted 100–300 fold in the assay, inhibition was produced only in the presence of reducing substrate. The activation of the DCIP activity observed with diaphorase was also produced by DPNH alone and hence is clearly a different phenomenon to the inhibition of the lip S_2 activity. The inhibitions were obtained very rapidly and were stable for several hours. After that they reversed slowly as the reducing substrate became exhausted through a slow reaction of the enzyme with O_2. The inhibitions were independent of the order of addition of the arsenite and substrates.

It is clear from the results presented here that arsenite inhibition is not *necessarily* a reaction with enzyme-bound lip$(SH)_2$ as postulated before[6–8,2] but that an arsenite-sensitive dithiol is involved in lipoyl dehydrogenase, which has been separated from the rest of the KGD complex[2,9], and shown not to contain any bound lipoic acid[2]. In view of the involvement of the flavin at the semiquinoid level[5] and the results presented here it is proposed that the reaction mechanism of lipoyl dehydrogenase may be represented as follows:

$$\text{FAD enz } S_2 + \text{lip } (SH)_2 \rightleftharpoons \text{FAD enz } (SH)_2 + \text{lip } S_2 \quad (1)$$

$$\text{FAD enz } (SH)_2 \rightleftharpoons \text{FADH}^{\bullet} \text{ enz } S^{\bullet}SH \quad (2)$$

$$\text{FADH}^{\bullet} \text{ enz } S^{\bullet} SH + \text{DPN} \rightleftharpoons \text{FADH}^{\bullet} \text{ enz } S_2 + \text{DPN}^{\bullet} + H^{+} \quad (3)$$

$$\text{FADH}^{\bullet} \text{enz } S_2 + \text{DPN} \rightleftharpoons \text{FAD enz } S_2 + \text{DPN}^{\bullet} + H^{+} \quad (4)$$

$$2\ \text{DPN}^{\bullet} + H^{+} \rightleftharpoons \text{DPNH} + \text{DPN} \quad (5)$$

$$\text{Overall: lip } (SH)_2 + \text{DPN} \rightleftharpoons \text{lip } S_2 + \text{DPNH} + H^{+} \quad (6)$$

In this formulation, it is proposed that the sharing of electrons between DPNH and DPN (reaction(5)) may be very much enhanced by the binding of these substances at adjacent groups in the enzyme, so that the near-planar pyridine rings lie closely parallel. This situation, similar to that proposed by GRABE[10] for electron transfer between pyridine nucleotide and flavin, would be expected to result in electron-sharing between the two enzyme-bound pyridine nucleotides.

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