Preliminary Notes

PN 10033

A new NAD$^+$-dependent spectral species of lipoamide dehydrogenase

Previous work[1,2] has shown that upon addition to lipoamide dehydrogenase (NADH: lipoamide oxidoreductase, EC 1.6.4.3) of NADH or reduced lipoamide the flavin absorption is decreased by about $^1/_3$ in the 450-mμ region, while at the same time a band in the region of 500–700 mμ is formed. This was attributed to the transfer of 2 electrons from the donor to the enzyme leading to reduction of the flavin and a S–S bridge with formation of a flavin semiquinone and a S· radical. It was shown that the band at 500–700 mμ belongs to a catalytically active intermediate of the overall reaction. SEARLS AND SANADI[3,4] have postulated that this intermediate is a

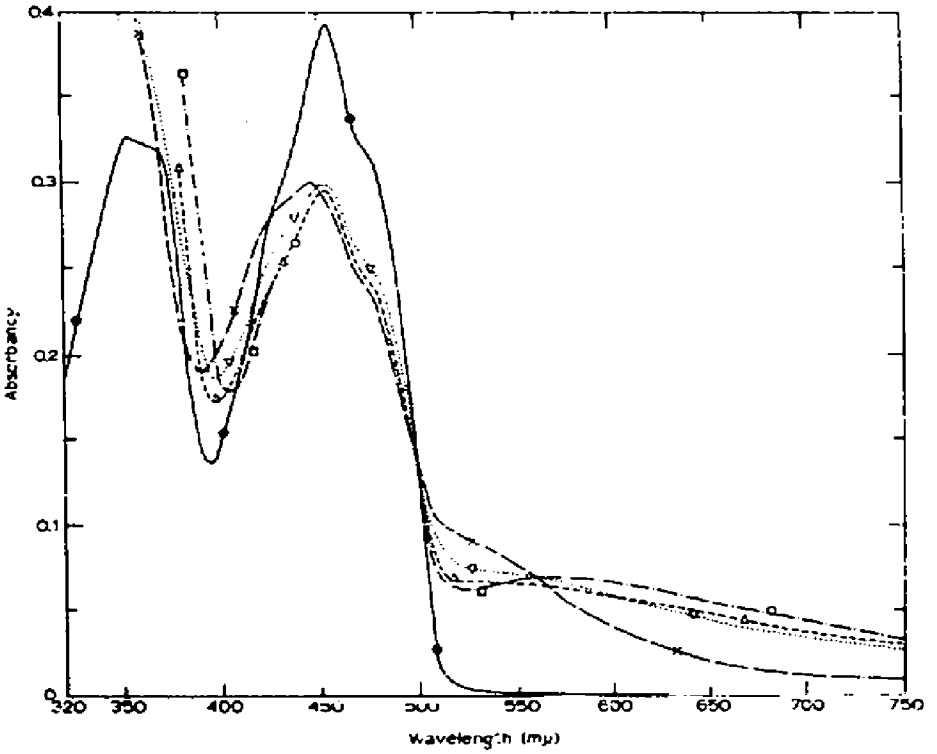

Fig. 1. The effect of the addition of different amounts of NAD$^+$ to the NADH-produced semiquinone of lipoamide dehydrogenase. The experiment was carried out in a volume of 3 ml in 0.05 M phosphate buffer (pH 7.6), 1 mM EDTA under anaerobic conditions. The spectra were recorded on a Cary Model-14 Recording Spectrophotometer 1 min after mixing with a scanning speed of 5 mμ/sec starting at 750 mμ. The reference cell contained water. The absorbancy is corrected for dilution caused by the various additions. The NAD$^+$ used was neutralized. ●—●, 0.104 μmole oxidized enzyme; ×···×, semiquinone produced by 0.3 μmole NADH; ▽—▽, 1 μmole NAD$^+$ added to semiquinone; △—△, 2.5 μmoles NAD$^+$ added to semiquinone; □—□, 10 μmoles NAD$^+$ added to semiquinone.

charge-transfer complex, but it has been shown that the experimental basis for their argument is not valid[5]. The reaction scheme proposed by SEARLS AND SANADI also differed from that proposed by us in many respects; *e.g.*, they proposed that the intermediate is formed by NADH via the fully reduced flavin, whereas we believe that the semiquinone is formed via a NAD^+-dependent intermediate[2]. More recent work[6,7] has favoured our interpretation rather than that of SEARLS AND SANADI.

We now wish to report the spectral demonstration of the existence of a NAD^+-dependent species of lipoamide dehydrogenase. Fig. 1 shows that upon the reduction of the oxidized enzyme by about 3 moles NADH per mole enzyme flavin in the presence of an excess of NAD^+ the maximum is at 453 mμ compared with 445 mμ obtained in the absence of added NAD^+. In the 500–750-mμ region the shoulder at about 530 mμ of the semiquinone obtained in the absence of added NAD^+ is replaced by a broad band with a maximum at about 565 mμ, and with a greater absorption than the semiquinone above about 560 mμ (Fig. 1). This greater absorption is not due to the charge-transfer complex, which develops when the enzyme becomes fully reduced in the presence of NAD^+ and which has its maximum absorption at 720 mμ (refs. 2, 7 and 8). The increased absorption at about 400–420 mμ in the semiquinone compared with the oxidized enzyme is less in the presence of NAD^+.

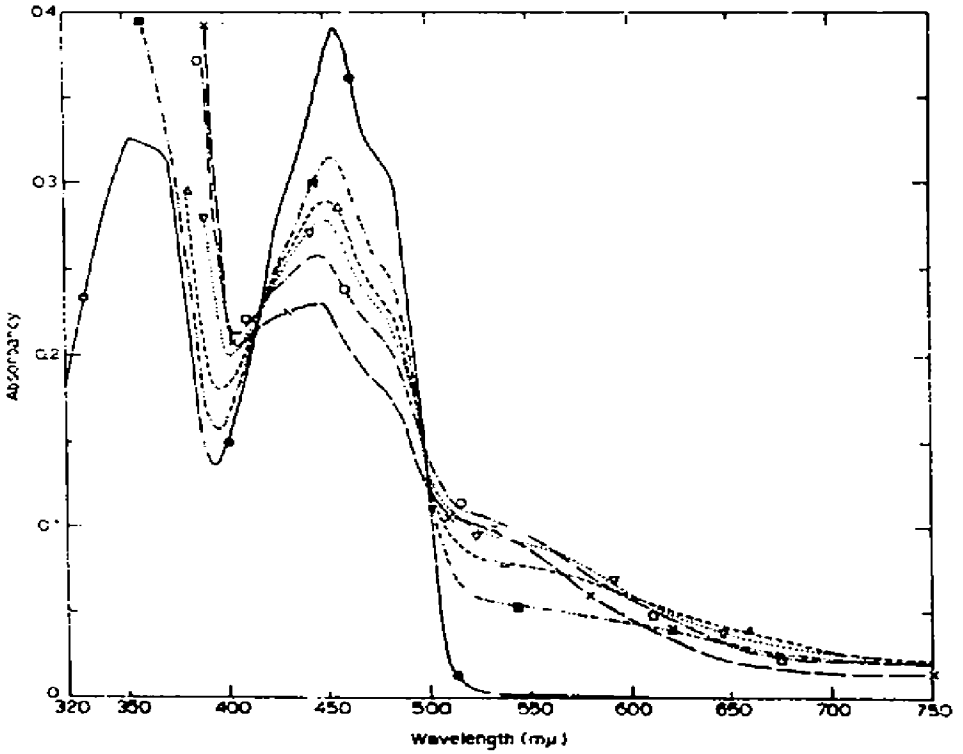

Fig. 2. The effect of the addition of different amounts of NADH to lipoamide dehydrogenase in the presence of 1 μmole NAD^+. Conditions were the same as described in Fig. 1. ●—●, 0.104 μmole oxidized enzyme; ■—■, addition of 0.2 μmole NADH; △—△, addition of 0.6 μmole NADH; ▽—▽, addition of 1 μmole NADH; □--□, addition of 2 μmoles NADH; ×—×, a control experiment in which the enzyme was reduced without added NAD^+ by 2 μmoles NADH.

Upon the addition of increasing amounts of NADH to the enzyme in the presence of excess NAD^+, the NAD^+-dependent intermediate with maximum at 565 mμ is converted into the semiquinone, with a shoulder at 530 mμ (Fig. 2). On the other hand, the addition of increasing amounts of NAD^+ to the NADH-reduced enzyme (semiquinone) leads to the conversion of the semiquinone into the 565-mμ intermediate (Fig. 1). Thus it is clear that the new intermediate is in reversible equilibrium with the previously described semiquinone.

The nature of the new spectral species must remain at the moment purely conjectural. The characteristic absorption appears to be caused specifically by NAD^+. Experiments similar to those of Fig. 1 but using NADPH as reductant gave the normal semiquinone spectrum; the further addition of $NADP^+$ led only to partial reoxidation without any new spectral bands appearing. Similar results were also obtained with reduced and oxidized nicotinamide–hypoxanthine dinucleotide (deamino-NAD).

Preliminary stopped-flow experiments carried out in collaboration with Professor Q. H. GIBSON show that the rate of formation of the new absorbing species is extremely rapid: its production is complete within the 3-msec dead time of the apparatus. Thus the new species may well be a catalytically important intermediate, possibly identical with States IV or VI of our previously postulated reaction mechanism[2]. Preliminary electron-spin-resonance experiments in collaboration with Dr. H. BEINERT and Dr. N. M. ATHERTON have shown that the reduced enzyme in the presence of NAD^+ gives a free-radical signal. However, the time course of development of the radical signal is very much slower than the production of the 565-mμ intermediate. This work will be reported in detail later.

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