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# LIPOAMIDE DEHYDROGENASE OF THE PHYTOPATHOGENIC FUNGI *PYTHIUM ULTIMUM* AND *PHYTOPHTHORA ERYTHROSEPTICA*

## DIFFERENCES IN CONFORMATION AND KINETICS

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SUMMARY

1. Lipoamide dehydrogenase (NADH: lipoamide oxidoreductase, EC 1.6.4.3) was isolated from two closely related phytopathogenic fungi, *Pythium ultimum* and *Phytophthora erythroseptica*. The enzymes of both microorganisms differ considerably in their catalytic properties, the lipoamide dehydrogenase of *P. ultimum* being much more active (catalytic centre activity estimated for the reduction of lipoate by NADH: 5900 $min^{-1}$; for the reduction of $NAD^+$ by reduced lipoamide: 100 000 $min^{-1}$) than that of *Ph. erythroseptica* (corresponding values, 720 $min^{-1}$ and 4200 $min^{-1}$). The Michaelis constants for both reactions were determined.

2. Under identical conditions the fluorescence intensity of the enzyme from *Ph. erythroseptica* at 20 °C is about three times higher than that of lipoamide dehydrogenase from *P. ultimum*. Upon anaerobic reduction with NADH, the *Phytophthora* enzyme shows beyond 500 nm the typical spectrum of the two equivalent reduced enzyme as reported for the pig heart enzyme (cf. Massey, V., Gibson, Q. H. and Veeger, C. (1960) Biochem. J. 77, 341–351 (ref. 1)), the *Pythium* enzyme becoming more bleached in the flavin region without being fully reduced.

3. At pH 7.6 in phosphate buffer, higher concentrations of $NAD^+$ inhibit the activity of the lipoamide dehydrogenase from both fungi when lipoate is reduced with NADH. At pH 5.9 (optimum), $NAD^+$ stimulates the reaction in the case of the *P. ultimum* enzyme, but inhibits the *Ph. erythroseptica* enzyme. The results indicate that both enzymes have two $NAD^+$ binding sites.

4. The results underline the strikingly high genetic variability within the fungus subfamily *Pythieae*.

## INTRODUCTION

The lower fungi *Pythium ultimum* and *Phytophthora erythroseptica* belong to the subfamily *Pythieae* of the *Peronosporaceae* within the class of *Phycomycetes*. Both species are phytopathogenic, *P. ultimum* as a potent soil saprophyte attacking a wide

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range of host plants; on the other hand, the facultative parasitism of *Ph. erythroseptica* is much less polyphag, the potato plant being the main host.

The *Pythieae*, and especially the genus *Phytophthora*, are known for their high physiological variability [2] .Results from comparative growth experiments and biochemical investigations with *P. ultimum* and *Ph. erythroseptica* led to the preliminary conclusion, that enzymes of "NADH diaphorase" character within these two species and other fungi of the genus *Phytophthora* might differ in their catalytic activities [3].

On the basis of this suggestion, it was decided to isolate the enzyme lipoamide dehydrogenase (NADH: lipoamide oxidoreductase, EC 1.6.4.3) from the two species under investigation. The enzyme has the character of a "NADH diaphorase" [4, 5]. Lipoamide dehydrogenase catalyzes the reduction of $NAD^+$ to NADH by the oxidative decarboxylation of pyruvate and $\alpha$-oxoglutarate. Accordingly, in metabolism it is localized within the multi-enzyme complexes of pyruvate dehydrogenase and $\alpha$-oxoglutarate dehydrogenase. Lipoamide dehydrogenase has been purified from different sources, such as pig heart [1], *Escherichia coli* [6], *Spinacia oleracea* [7], *Saccharomyces cerevisiae* [8] *and Azotobacter vinelandii* [9].

In this work, an attempt was made to characterize the lipoamide dehydrogenase of *P. ultimum* and *Ph. erythroseptica* by way of absorption spectra in different states of reduction, by fluorescence spectra and by kinetic investigations.

## MATERIALS AND METHODS

*P. ultimum* was obtained from the American Type Culture Collection, *Ph. erythroseptica* from the Bureau voor Schimmelcultures, Baarn, The Netherlands. Both fungi were raised on a large scale in a fermenter by Boehringer and Soehne, Tutzing/Obb., Germany. The nutrient solution was as follows: 20 g glucose; 1 g $KH_2PO_4$; 1.8 g L-glutamic acid; 0.5 g $MgSO_4 \cdot 7H_2O$; 0.1 g NaCl; 0.1 g $CaCl_2 \cdot 2H_2O$; 5 mg $FeCl_3 \cdot 6H_2O$; 0.1 mg thiamine in 1 l of distilled water; pH 5.5 with NaOH. After harvest the mycelium was squeezed out between filter paper, deep-frozen and lyophilized. Until use, the mycelium was stored at +4 °C. No material older than six weeks was used as a source for enzyme isolation.

Standard enzyme activity with reduced lipoamide as hydrogen donor and $NAD^+$ as acceptor was determined in incubation mixtures containing 0.05 M phosphate buffer, pH 7.6; 0.07% (w/v) bovine serum albumin; 1 mM EDTA; 0.17 mM $NAD^+$; 60 $\mu$M reduced lipoamide; enzyme solution. The reaction was started by the addition of an amount of enzyme giving an increase of absorbance at 340 nm not exceeding 0.06 absorbance units per min. The standard assay of the reverse reaction, using NADH as donor and lipoate as hydrogen acceptor was carried out following the procedure described by Massey et al. [1]. In some experiments, the citrate buffer of the standard test was replaced by 0.1 M phosphate buffer (final concn) at the desired pH. Unless otherwise stated, all tests were carried out at pH 5.9. Protein determinations were made by the micro-biuret method [10], using ovalbumin as a standard.

The enzyme concentration of purified samples was calculated from the absorption at 454 nm, with the assumption that the extinction coefficient of enzyme-bound FAD is the same as that of free FAD (11 300 $M^{-1} \cdot cm^{-1}$) and that two molecules of FAD are bound by one dimer molecule of enzyme. Turnover numbers are expressed as moles of substrate oxidized or reduced per mole of FAD per min.

Absorption spectra were recorded on a Cary 14 spectrophotometer; fluorescence spectra (not corrected for photomultiplier sensitivity) were recorded on a Hitachi–Perkin–Elmer MPF-2a spectrofluorimeter. Reduced lipoamide was prepared by the procedure of Reed et al. [11]. Most of the fine chemicals used were purchased at Boehringer and Soehne, Mannheim; lipoate and oxidized lipoamide were obtained from Fluka; bovine serum albumin from Behringwerke, Marburg; ovalbumin from Serva.

## RESULTS

### *Enzyme purification*

180-g samples of lyophilized mycelium were homogenized in a mechanically driven mortar together with 300 g of dry sand for a few minutes until the fungus material had the consistency of wheat flour. Another 300 g of sand and 380 ml of 0.03 M phosphate buffer (pH 7.6) containing 0.3 mM EDTA were added stepwise and the grinding procedure continued for 2 h. Then another 380 ml of buffer were added, and mixed with the homogenized material in the mortar for a short time. After centrifugation at 2200 $\times$ *g* for 30 min (save supernatant), the residue was washed with buffer and centrifuged. The combined supernatants were stirred overnight in a coldroom, followed by centrifugation at 23 000 $\times$ *g* for 30 min. The supernatant was saved.

After producing a slurry from the residue with buffer, this was subjected to ultrasonification under constant cooling (25-ml samples, three times, 2 min each) and subsequent mechanical stirring overnight. After centrifuging the slurry at 23 000 $\times$ *g* for 30 min, the residue was discarded, and the supernatants combined. The yellow-brownish extract was adjusted to pH 6.2 with acetic acid. Subsequently, 2% protamine sulphate solution was added stepwise during about half an hour to 22% of the original volume of the extract. After stirring for another 15 min the mixture was centrifuged for 30 min at 23 000 $\times$ *g* and the residue discarded. After adjusting to pH 7.6 again, the lipoamide dehydrogenase was precipitated with solid $(NH_4)_2SO_4$ between 50 and 70% saturation and further purified according to Massey et al. [1] by cellulose-calcium phosphate chromatography and $(NH_4)_2SO_4$ precipitation. The enzyme was dissolved in 1–2 ml of 0.03 M phosphate buffer (pH 7.6). In a subsequent step lipoamide dehydrogenase was further purified by gel chromatography on a Sephadex G-200 column, 40 cm $\times$ 2.5 cm. The most active fractions were pooled, concentrated by $(NH_4)_2SO_4$ precipitation, finally subjected to a heat treatment (cf. ref. 1) (5 min at 70 °C) and centrifuged again. By this last step a peak in the absorption spectrum at 410 nm was removed. The absorption spectra in the visible region of the purified enzymes were completely identical with that of pig heart lipoamide dehydrogenase (cf. ref. 1). The absorbance ratio 280 nm/454 nm was 6.6 for the enzyme from *Ph. erythroseptica*, and 9.0 for the enzyme from *P. ultimum*.

The procedure for the purification of the *Ph. erythroseptica* lipoamide dehydrogenase is summarized in Table I. The enzyme from *P. ultimum* showed on Sephadex G-200 and polyacrylamide gel electrophoresis a molecular weight of approximately 108 000.

The pH optimum for the reduction of $NAD^+$ by reduced lipoamide is about 7.8 in 50 mM phosphate buffer; on the other hand, the optimal pH value for the reduction of lipoate by NADH is in 0.8 M citrate buffer about 5.9, as compared with

TABLE I

PURIFICATION OF LIPOAMIDE DEHYDROGENASE FROM *PHYTOPHTHORA ERYTHROSEPTICA*[*]

| Fraction | Volume (ml) | Protein (mg) | Specific activity[**] (nmoles/mg protein) | Recovery (%) |
|---|---|---|---|---|
| 1. Crude extract from 650 g of lyophilized mycelium | 4000 | 83 000 | 0.85 | 100 |
| 2. Protamine sulphate step, supernatant | 4350 | — | — | — |
| 3. 50–70% $(NH_4)_2SO_4$ precipitate, redissolved in buffer | 360 | 3 650 | 12.9 | 67 |
| 4. Eluate from calcium phosphate gel column after 50–70% $(NH_4)_2SO_4$ precipitation | 2.5 | 298 | 100 | 42 |
| 5. Most active fractions after Sephadex G-200 gel chromatography, after concentration by $(NH_4)_2SO_4$ precipitation | 1.5 | 21.7 | 1020 | 31 |
| 6. After 5 min/70 °C heat treatment | 1.5 | 18.1[***] | 1240 | 31 |

[*] As averaged from several runs.

[**] As determined by the reaction $lipS_2 + NADH + H^+ \rightarrow NAD^+ + lip(SH)_2$ at pH 6.35 (citrate buffer). $LipS_2$, oxidized lipoate; $lip(SH)_2$, reduced lipoate.

[***] According to the absorption spectrum this fraction contained 12.2 mg of pure lipoamide dehydrogenase (totally).

5.65 for the enzyme from pig heart [1, 12]. The pH optima of the enzyme from both fungi are identical. In the case of lipoate reduction, in the presence of 0.1 M phosphate buffer in the incubation mixture, the reaction runs at 30% of the rate in citrate buffer.

In the two standard tests (see under Materials and Methods), the enzymes of the two microorganisms differ considerably in their activity. With the lipoamide dehydrogenase from *P. ultimum*, the standard activity in the reduction of lipoate by NADH is about 18 times higher than with the enzyme from *Ph. erythroseptica* (8400 and 650 $min^{-1}$, respectively). The corresponding factor for the reduction of $NAD^+$ by reduced lipoamide is 8 (1840 and 148 $min^{-1}$); the *Pythium* enzyme catalyzing at a higher rate than the one from the other fungus.

*Absorption and fluorescence spectra*

The visible spectrum of the lipoamide dehydrogenase of *P. ultimum* and *Ph. erythroseptica* is shown in Fig. 1. The oxidized spectra are identical for both enzymes, and identical to that of the oxidized form of the lipoamide dehydrogenase from pig heart. Upon the anaerobic addition of excess of NADH, the enzyme from *Ph. erythroseptica* behaves just as the pig heart enzyme does: the stable red form (half or two-electron reduced form) is produced. On further addition of solid dithionite, the fully reduced enzyme is obtained. Anaerobic reduction of the *Pythium* lipoamide dehydrogenase by an excess of NADH, on the other hand, leads to a slightly different spectrum. As compared with the enzyme from *Ph. erythroseptica*, there is some bleaching in the region of the FAD absorption. At 530 nm and higher wavelengths, however, the absorption of both lipoamide dehydrogenases is much the same. Neither the

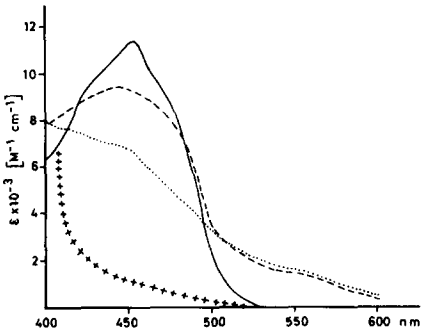


Fig. 1. Absorption spectra of lipoamide dehydrogenase from *Ph. erythroseptica* and *P. ultimum* in 0.03 M phosphate buffer (pH 7.6) at 25 °C. ———, oxidized lipoamide dehydrogenase, spectrum identical for the enzyme of both fungi; — — —, half-reduced form (two-electron state) of the enzyme from *Ph. erythroseptica*, produced spontaneously after the anaerobic addition of 5.2 moles of NADH per mole of FAD. Further addition of NADH did not change the spectrum; ·····, spectrum of the enzyme from *P. ultimum*, formed spontaneously after the anaerobic addition of 4.5 moles of NADH per mole of FAD. Further addition of NADH did not change the spectrum; +++, fully reduced form (four-electron state) of the enzyme from both fungi, produced spontaneously after the addition of solid dithionite.

*Pythium* enzyme nor the one from *Ph. erythroseptica* exhibits any absorption in the region between 640 and 700 nm.

The fluorescence emission spectrum of lipoamide dehydrogenase from *P. ultimum*, obtained after excitation at 366-nm wavelength at room temperature is almost identical with that of FAD (Fig. 2), except that the emission peak of the *Pythium* enzyme is shifted to about 515 nm compared with free FAD (525 nm). The fluorescence intensities of this enzyme and of free FAD are about the same. The lipoamide dehydrogenase from *Ph. erythroseptica*, on the other hand, exhibits a considerably greater fluorescence intensity than FAD (about three times higher than that of the

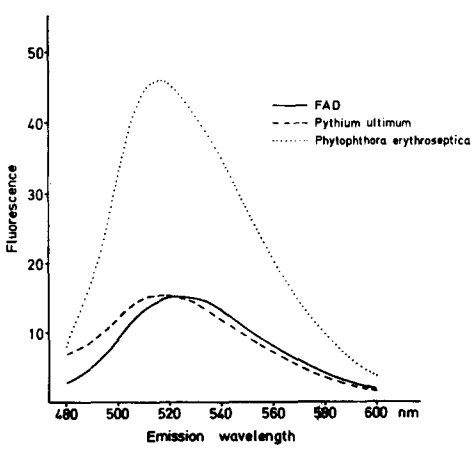


Fig. 2. Fluorescence emission spectra of FAD and lipoamide dehydrogenase from *P. ultimum* and *Ph. erythroseptica*, respectively, in 0.15 M phosphate buffer (pH 7.6) at 25 °C. FAD concentration in all cases, 6.8 $\mu$M; excitation at 366 nm.

free prosthetic group). The shift in emission maximum is identical with that of the other enzyme. The fluorescence of pig heart lipoamide dehydrogenase [13] is about 3–4 times higher than that of FAD under these conditions.

*Kinetic studies*

*The reduction of $NAD^+$ by reduced lipoamide.* It is not possible to derive with certainty from the Lineweaver–Burk plots of the kinetics of this reaction (which actually is the physiological process occurring within the hyphae of the fungi) whether the lines are parallel or slightly converging. Thus it is not possible to form a definite conclusion about the actual mechanism of lipoamide dehydrogenase, i.e. a ping-pong mechanism as has been proposed [1, 13] or opposed [14–17] on different grounds. Only in the case of lipoamide dehydrogenase from *A. vinelandii* was it possible to conclude with certainty that the reaction is catalyzed along a ternary complex mechanism [9, 18]. Good competitive inhibitors used in other studies (cf. refs 19 and 20) which are necessary to discriminate between the possible mechanisms are not known.

The catalytic centre activity at infinite concentration of both substrates has been calculated from the kinetic plots (Fig. 3). There is an almost 25-fold difference

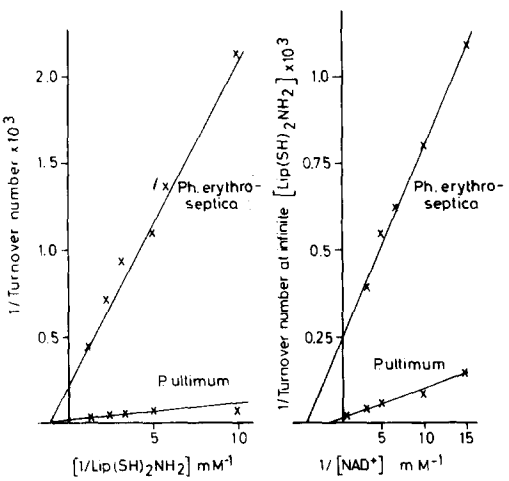


Fig. 3. Left: effect of reduced lipoamide at different concentrations in the assay system on the maximal catalytic centre activity. Right: effect of $NAD^+$ at different concentrations in the assay system on the maximal catalytic centre activity. These figures were obtained from the extrapolation of the kinetic data to infinite acceptor (left) and donor (right) concentrations.

in the values of the two fungal enzymes: $V$ of *P. ultimum* lipoamide dehydrogenase was estimated to be 100 000, $V$ of the enzyme from *Ph. erythroseptica* to be only 4200 moles $NAD^+$ reduced per mole of FAD per min. Such striking differences were not found for the Michaelis constants (for details see Table II).

*Kinetics of the reduction of oxidized lipoate by NADH.* The reaction

$$lipS_2 + NADH + H^+ \rightarrow lip(SH)_2 + NAD^+$$

TABLE II

PROPERTIES OF LIPOAMIDE DEHYDROGENASE FROM DIFFERENT SOURCES

| Property | Lipoamide dehydrogenase from | | | | |
|---|---|---|---|---|---|
| | *Pythium ultimum* | *Phytophthora erythroseptica* | Pig heart [1], [12] | *Saccharomyces cerevisiae* [16] | *Azotobacter vinelandii* [8, 9] |
| pH optimum for reaction | | | | | |
| $lipS_2 \leftarrow NADH$ | 5.9 | 5.9 | 5.65 | – | 6.5 |
| $lip(SH)_2NH_2 \rightarrow NAD^+$ | 7.8 | 7.8 | 7.9 | – | 7.8 |
| Fluorescence intensity as compared with that of FAD at 515-nm emission wavelength, in 0.15 M phosphate buffer at pH 7.6 | identical (22 °C) | 3-fold (22 °C) | 4-fold (12 °C) | – | 4-fold (20 °C) |
| $V$ ($min^{-1}$) in reaction | | | | | |
| $lipS_2 \leftarrow NADH$ | 5 900 | 720 | 4 000 | 5 000 | 6 800 |
| $lip(SH)_2NH_2 \rightarrow NAD^+$ | 100 000 | 4200 | 17 300 | 26 000 | 18 500 |
| $K_m$ (mM) in reaction | | | | | |
| $lipS_2 \leftarrow NADH$ (for $lipS_2$) | 1 | 2 | 2 | 0.9 | – |
| $lip(SH)_2NH_2 \rightarrow NAD^+$ | | | | | |
| (for $lip(SH)_2NH_2$) | 1 | 0.8 | 0.25 | 0.7 | 0.15 |
| (for $NAD^+$) | 0.9 | 0.2 | 0.36 | 0.4 | 0.18 |

$lipS_2$, oxidized lipoate; $lip(SH)_2NH_2$, reduced lipoamide.

(where $lipS_2$ and $lip(SH)_2$ are oxidized and reduced lipoic acid, respectively) is inhibited by moderate concentrations of NADH in the presence of citrate in the assay mixture (Fig. 4). No striking difference in the extent of inhibition was observed with the two fungal enzymes. At 40 $\mu$M NADH and infinite lipoate concentration the activity is about 720 for the enzyme from *Ph. erythroseptica*, and about 5900 for the lipoamide dehydrogenase from *P. ultimum*. Values for the Michaelis constants are shown in Table II.

With commercial samples of NADH, the reaction shows an initial lag-phase,

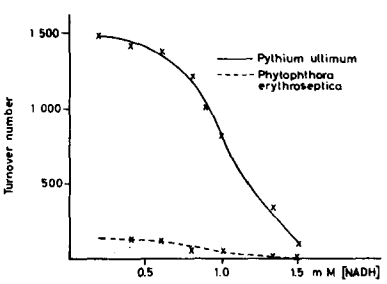


Fig. 4. Inhibition of the reaction $lipS_2 + NADH + H^+ \rightarrow lip(SH)_2 + NAD^+$ by NADH at different concentrations in the incubation mixture. Temperature 25 °C; standard assay condition with citrate buffer with the NADH concentration indicated. $lipS_2$, oxidized lipoate; $lip(SH)_2$, reduced lipoate.

when pig heart lipoamide dehydrogenase is used as the enzyme. As has been elucidated by Massey and Veeger [21, 22], this lag-period is due to the absence of $NAD^+$ at the beginning of the reaction. For the reduction of lipoic acid by NADH, $NAD^+$ is needed as a co-factor. Its stepwise formation after starting the reaction, enhances the turnover number gradually. Once $NAD^+$ is destroyed by $NAD^+$ nucleosidase present in the mixture, no reduction of lipoate takes place.

The same phenomenon can be observed with the lipoamide dehydrogenases of *P. ultimum* and *Ph. erythroseptica* (Fig. 5). The time course of the reaction in citrate

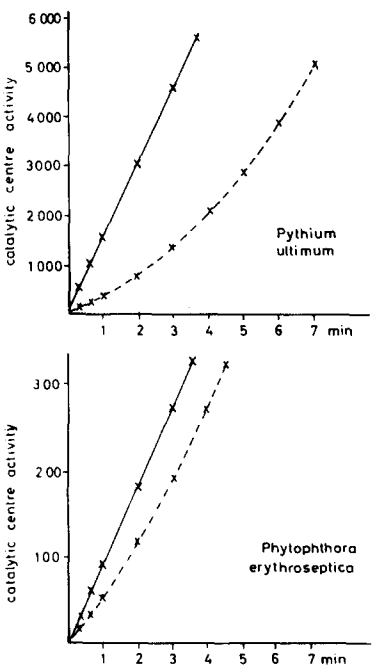


Fig. 5. Effect of $NAD^+$ on the reaction rate during the reduction of lipoate by NADH catalyzed by lipoamide dehydrogenase from *P. ultimum* and *Ph. erythroseptica*, respectively. Standard assay conditions; temperature, 25 °C; citrate buffer. ———, reaction with addition of 0.1 mM $NAD^+$; – – –, reaction without addition of $NAD^+$.

buffer gives a straight line when $NAD^+$ is added together with the other substrates. The lag-period observed in the absence of $NAD^+$ is, however, much more pronounced with the *Pythium* enzyme than with the *Phytophthora* one. Thus lipoate reduction with lipoamide dehydrogenase from *P. ultimum* depends much more on $NAD^+$ as a co-factor than that by the enzyme from the other fungus. A similar dependence of the reverse reaction on the $NAD^+$ concentration can be found in Table II, where it is shown that the $K_m$ for $NAD^+$ is about five times higher for the *Pythium* enzyme than for *Phytophthora*. Furthermore, Fig. 6 shows that the *Pythium* enzyme reaches full activity at a $NAD^+$/NADH ratio of 5–7. Both enzymes do not show product inhibition, at a 1 mM $NAD^+$ concentration when citrate buffer at the optimal pH (5.9) was used throughout.

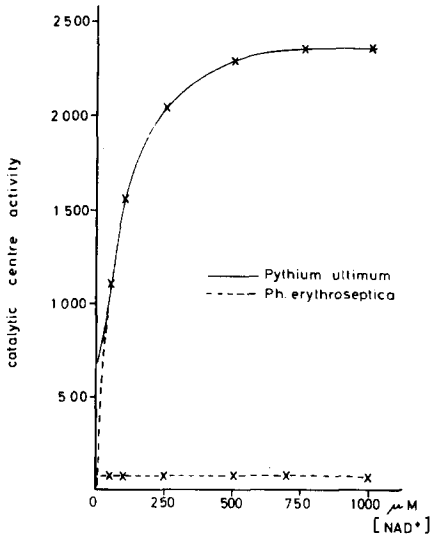


Fig. 6. Effect of different $NAD^+$ concentrations on the reaction $lipS_2 + NADH + H^+ \rightarrow lip(SH)_2 + NAD^+$, using lipoamide dehydrogenase from *P. ultimum* and *Ph. erythroseptica*. Standard assay conditions in citrate buffer at pH 5.9. $lipS_2$, oxidized lipoate; $lip(SH)_2$, reduced lipoate.

However, when citrate is replaced by phosphate of the same pH (Fig. 7) 1 mM $NAD^+$ inhibits the *Phytophthora* enzyme depending on the NADH concentration. Furthermore NADH does not show substrate inhibition. In contrast the *Pythium* enzyme shows substrate inhibition by NADH in phosphate buffer, pH 5.9, while the activity is enhanced considerably by $NAD^+$. Depending on the NADH concentration used, this activation is followed by inhibition.

In phosphate buffer, pH 7.6, the situation is quite different (Fig. 8). The enzyme from both organisms is inhibited by much lower $NAD^+$ concentrations. Upon replotting the inhibition pattern according to the method of Dixon and Webb [23] a

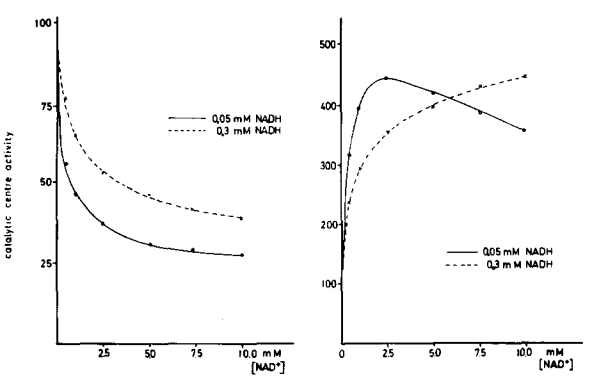


Fig. 7. Influence of $NAD^+$ at different concentrations on the reaction $lipS_2 + NADH + H^+ \rightarrow lip(SH)_2 + NAD^+$, employing 0.05 M phosphate buffer at pH 5.9 in the medium. Temperature, 25 °C. Left: *Ph. erythroseptica* enzyme. Right: *P. ultimum* enzyme. $lipS_2$, oxidized lipoate; $lip(SH)_2$, reduced lipoate.

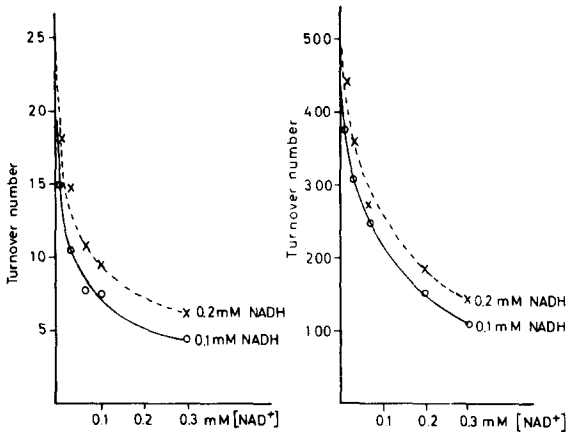


Fig. 8. Inhibition of the reaction $lipS_2 + NADH + H^+ \rightarrow lip(SH)_2 + NAD^+$ by $NAD^+$ at different concentrations, using 50 mM phosphate buffer at pH 7.6 in the medium. Temperature, 25 °C. Left: *Ph. erythroseptica* enzyme. Right: *P. ultimum* enzyme. $lipS_2$, oxidized lipoate; $lip(SH)_2$, reduced lipoate.

complex inhibition pattern becomes clear (Fig. 9), because curved plots are obtained, ruling out pure product inhibition. It cannot be concluded with certainty, whether the inhibition is competitive or non-competitive. At least at low concentrations, $NAD^+$ seems to inhibit competitively with respect to NADH in both enzymes. The

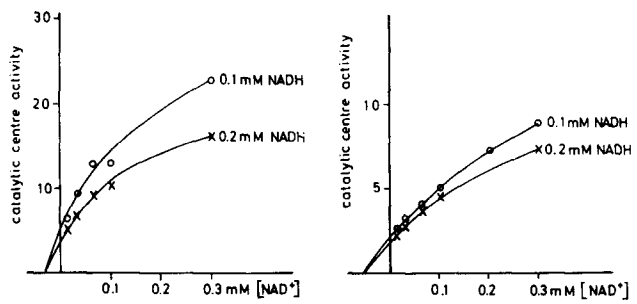


Fig. 9. Dixon plot drawn from data in Fig. 8. Left: *Ph. erythroseptica* enzyme. Right: *P. ultimum* enzyme.

deviation from linearity clearly indicates the existence of a second $NAD^+$ site. It must be recalled that the pig heart enzyme also has two $NAD^+$ binding sites [24]. Furthermore in phosphate buffer, pH 7.6, NADH does not show substrate inhibition with both fungal enzymes at pH 7.6 as is found in citrate buffer (pH 5.9) or in phosphate buffer (pH 5.9) with the *Pythium* enzyme alone.

## DISCUSSION

As compiled in Table II, there is a large difference in the catalytic properties of the lipoamide dehydrogenase from *P. ultimum* and *Ph. erythroseptica*. Actually,

the catalytic properties of lipoamide dehydrogenase from yeast, *Azotobacter* and pig heart are much more comparable than those of the two closely related fungi. This strongly supports the former observation of an unusually high biological variability within the fungus subfamily *Pythieae* [2]. Uhlenhaut [25] recently demonstrated the existence in five different *Pythium* species of different number and combinations of six isoenzymes of malate dehydrogenases and five isoenzymes of lactate dehydrogenase.

As can be concluded from the large difference in the fluorescence emission intensity of the flavin of the *Pythium* enzyme, its micro-environment differs considerably from that of the enzymes from *Phytophthora*, *Azotobacter* and pig heart. Recent evidence shows that the increase in fluorescence of the *Phytophthora*, *Azotobacter* and pig heart enzymes was related to an open FAD conformation, e.g. an FMN-like conformation [26] which is essential for the conformation–activity relation of the enzyme. The studies do not exclude the possibility that the quenching of the FAD fluorescence of the *Pythium* enzyme is caused by the protein. If so, it is remarkable that the quantum yield of the protein-bound FAD is the same as that of free FAD and that this enzyme has a much higher catalytic centre activity than the enzyme isolated from other sources (Table II).

In the case of *Ph. erythroseptica* the spectrum obtained upon anaerobic reduction is nearly identical with the spectrum of the stable two-equivalent reduced state of pig heart lipoamide dehydrogenase [1]. The spectrum obtained with the *Pythium* enzyme is similar to those reported for the lipoamide dehydrogenases from spinach [27] and *S. cerevisiae* [10]. Under the same conditions, the enzyme of *E. coli* is shifted to a nearly fully reduced state [28 ,29]. Apparently, after the anaerobic reduction of lipoamide dehydrogenase from *P. ultimum* by NADH a reduction state is obtained, which is intermediate between the half- and the fully-reduced state. It has been shown [30] for the pig heart enzyme, that increased absorbance at wavelengths beyond 500 nm occurs upon reduction of the enzyme with four reducing equivalents. It is open to discussion whether such a difference in the state of reduction is responsible for the striking differences in catalytic activity.

Massey and Veeger [22] postulated the existence of one binding site for NADH in the catalytic centre and another binding site Y for $NAD^+$ (or NADH, respectively), a postulate confirmed by the discovery of two spectral complexes [14, 24] belonging to two $NAD^+$ binding sites. The data presented here indicate that more than one $NAD^+$ binding site is also present in both fungal enzymes. Whether the binding of $NAD^+$ to the fungal enzymes leads, as with the pig heart enzyme [24], to the dissociation of a proton from the dimeric enzyme, and the dissociation into a monomeric protein, needs investigation.

As pointed out in Results, it is difficult to make a definite statement about the kinetic mechanism of the enzyme. Due to the high $K_m$ values for reduced lipoamide of the fungal enzymes it is not possible to investigate whether at much higher donor concentrations also here, as with the pig heart enzyme [14, 15], deviation from a parallel-line relationship, occurs. Although by a number of observations [14–16, 18] the ternary complex is favoured as the mode of catalytic mechanism, recent other evidence [31] argues against it. Part of the discrepancies could be due to the complex manner of $NAD^+$ binding [24] and its influence on the rate of the reaction [15, 32] which makes an unambiguous kinetic interpretation difficult to achieve.

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REFERENCES

1 Massey, V., Gibson, Q. H. and Veeger, C. (1960) Biochem. J. 77, 341–351
2 Erwin, D. G., Zentmyer, G. A., Galindo, J. and Niederhauser, J. S. (1963) Annu. Rev. Phytopathol. 1, 375–396
3 Fehrmann, H. (1971) Phytopathol. Z. 70, 230–262
4 Massey, V. (1958) Biochim. Biophys. Acta 30, 205–206
5 Veeger, C. and Massey, V. (1960) Biochim. Biophys. Acta 37, 181–183
6 Koike, M., Shah, P. C. and Reed, L. J. (1960) J. Biol. Chem. 235, 1939–1943
7 Basu, D. R. and Burma, D. P. (1960) J. Biol. Chem. 235, 509–513
8 Wren, A. and Massey, V. (1965) Biochim. Biophys. Acta 110, 329–336
9 Van den Broek, H. W. J. (1971) Ph. D. Thesis, Agricultural University, Wageningen, The Netherlands
10 Itzhahi, R. F. and Gill, D. M. (1964) Anal. Biochem. 9, 401–410
11 Reed, L. J., Koike, M., Levitch, M. E. and Leach, F. R. (1959) J. Biol. Chem. 232, 143–158
12 Massey, V. (1963) in The Enzymes (Boyer, P. D., Lardy, H. and Myrbäck, K., eds), 2nd edn. Vol. 7, pp. 275–306, Academic Press, New York
13 Wren, A. and Massey, V. (1966) Biochim. Biophys. Acta 122, 436–449
14 Visser, J., Voetberg, H. and Veeger, C. (1970) in Pyridine nucleotide-dependent enzymes (Sund, H., ed.), pp. 359–373, Springer-Verlag, Heidelberg
15 Visser, J., Havekes, L. and Veeger, C. (1972) Z. Naturforsch. 27, 1063–1066
16 Veeger, C., Voetberg, H., Pronk, J. and Visser, A. J. W. G. (1972) in Structure and Function of Oxidation Reduction Enzymes, (Åkeson, A. and Ehrenberg, A., eds), pp. 475–494, Pergamon Press, Oxford
17 Veeger, C. and Massey, V. (1963) Biochim. Biophys. Acta 67, 679–681
18 Veeger, C., Krul, J., Bresters, T. W., Haaker, H., Wassink, J. H., Santema, J. S. and De Kok, A. (1972) in Enzymes, Structure and Function (Drenth, J., Oosterbaan, R. A. and Veeger, C., eds), Proc. 8th FEBS Meeting, pp. 217–234, North Holland-Elsevier, Amsterdam
19 Koster, J. F. and Veeger, C. (1968) Biochim. Biophys. Acta 151, 11–19
20 Staal, G. E. J. and Veeger, C. (1969) Biochim. Biophys. Acta 185, 49–62
21 Massey, V. and Veeger, C. (1960) Biochim. Biophys. Acta 40, 184–185
22 Massey, V. and Veeger, C. (1961) Biochim. Biophys .Acta 48, 33–47
23 Dixon, M. and Webb, E. C. (1964) Enzymes, 2nd edn, Longmans, pp. 328–330, Green and Co., London
24 Van Muiswinkel-Voetberg, H. and Veeger, C. (1973) Eur. J. Biochem. 33, 285–291
25 Uhlenhaut, H. (1969) Ph. D. Thesis University of Bonn
26 Visser, A. J. W. G., Grande, H. J., Müller, F. and Veeger, C. (1974) Eur. J. Biochem., in the press
27 Matthews, J. and Reed, L. J. (1963) J. Biol. Chem. 238, 1869–1876
28 Koike, M., Shah, P. C. and Reed, L. J. (1960) J. Biol. Chem. 235, 1939–1943
29 Williams, Jr, C. H. (1965) J. Biol. Chem. 240, 4793–4800
30 Veeger, C., Dervartanian, D. V., Kalse, J. F., De Kok, A. and Koster, J. F. (1966) in Flavins and Flavoproteins (Slater, E. C., ed.), pp. 242–262, Elsevier, Amsterdam
31 Reed, J. K. (1973) J. Biol. Chem. 248, 4834–4839
32 Tsai, C. S. (1973) Arch. Biochem. Biophys. 159, 453–457