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Lipoamide dehydrogenase from *Azotobacter vinelandii*: site-directed mutagenesis of the His450-Glu455 diad

Kinetics of wild-type and mutated enzymes

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Three amino acid residues in the active site of lipoamide dehydrogenase from *Azotobacter vinelandii* were replaced with other residues. His450, the active-site base, was replaced with Ser, Tyr or Phe. Pro451, from X-ray analysis found to be in *cis* conformation positioning the backbone carbonyl of His450 close to N3 of the flavin, was changed to Ala. Glu455, from X-ray analysis expected to be involved in modulating the pK_a of the base (His450), was replaced with Asp and Gln. The general conclusion is that mutation of the His-Glu diad impairs intramolecular electron transfer between the disulfide/dithiol and the $FADH^-$/FAD.

The wild-type enzyme functions according to a ping-pong mechanism in the physiological reaction in which the formation of NADH is rate-limiting. Above pH 8.0 the enzyme is strongly inhibited by the product NADH. The pH dependence of the steady-state kinetics using the NAD^+ analog 3-acetylpyridine adenine dinucleotide ($AcPyAde^+$) reveals a pK_a of 8.1 in the pK_m $AcPyAde^+$ plot indicating that this pK_a is related to the deprotonation of His450 [Benen, J., Berkel van, W., Zak, Z., Visser, T., Veeger, C. & Kok de, A. (1991) *Eur. J. Biochem. 202*, 863–872] and to the inhibition by NADH.

The mutations considerably affect turnover. Enzymes with the mutations Pro451 → Ala, His450 → Phe and His450 → Tyr appear to be almost inactive in both directions. Enzyme His450 → Ser is minimally active, V at the pH optimum being 0.5% of wild-type activity in the physiological reaction. Rapid reaction kinetics show that for the His450-mutated enzymes the reductive half reaction using reduced 6,8-thioctic acid amide [$Lip(SH)_2$] is rate-limiting and extremely slow when compared to the wild-type enzyme. For enzyme Pro451 → Ala it is concluded that the loss of activity is due to over-reduction by $Lip(SH)_2$ and NADH. The Glu455-mutated enzymes are catalytically competent but show strong inhibition by the product NADH (enzyme Glu455 → Asp more than Glu455 → Gln). The inhibition can largely be overcome by using $AcPyAde^+$ instead of NAD^+ in the physiological reaction.

The rapid reaction kinetics obtained for enzymes Glu455 → Asp and Glu455 → Gln deviate from the wild-type enzyme. It is concluded that this difference is due to cooperativity between the active sites in this dimeric enzyme. Rapid reaction kinetics of enzymes His450 → Ser and Glu455 → Gln show the existence of two intermediates at the two-electron reduced level: a species with the NAD^+ bound, the flavin reduced and the disulfide intact (oxidized) and a species with NAD^+ bound, the disulfide reduced and the flavin oxidized. No spectral evidence is obtained for the participation of a proposed flavin C4a adduct intermediate [Thorpe, C. & Williams, C. H. Jr (1981) *Biochemistry 20*, 1507–1513] in the reaction mechanism of enzymes His450 → Ser and Glu455 → Gln.

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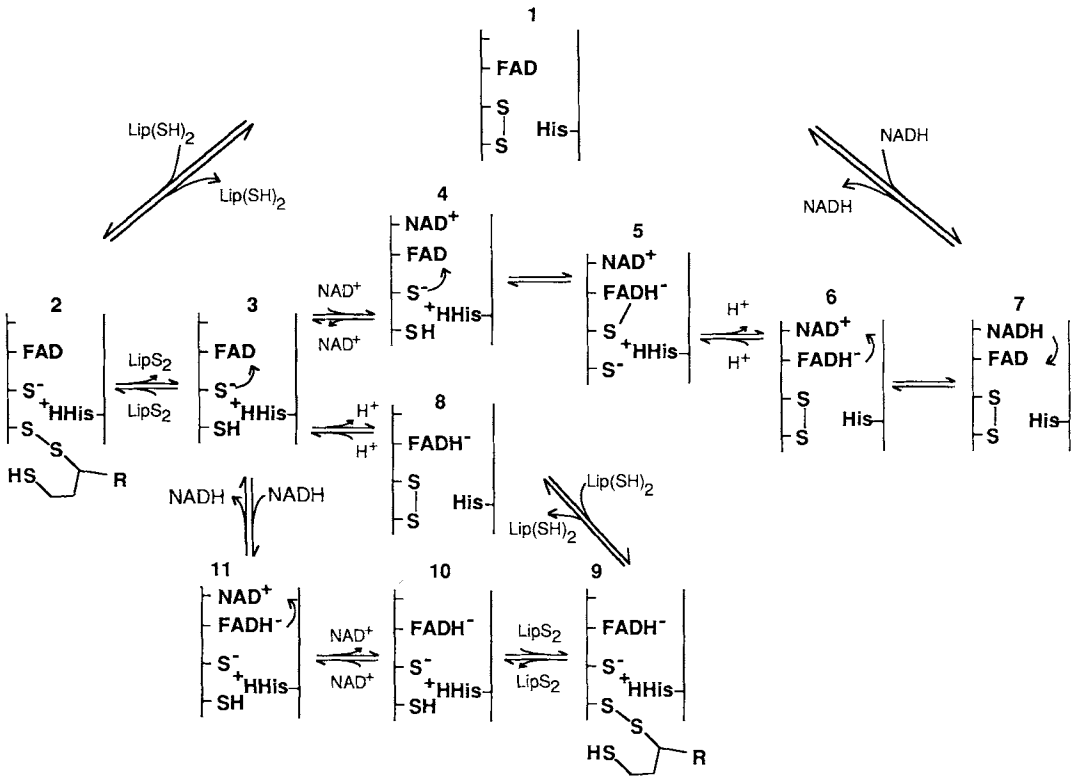
Abbreviations. E_{ox}, oxidized (mutated) lipoamide dehydrogenase; EH_2, two-electron-reduced (mutated) lipoamide dehydrogenase with disulfide reduced (530-nm charge-transfer species and tautomeric species); EH^-, EH_2 one-step deprotonated; E^{2-}, EH_2 two-steps deprotonated; EH_4, general descriptive term for four-electron-reduced (mutated) lipoamide dehydrogenase; $FADH^-$, reduced flavin; FlH^-, two-electron-reduced species with electrons on the flavin; $LipS_2$, DL-6,8-thioctic acid amide, lipoamide; $Lip(SH)_2$, reduced lipoamide; Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); $AcPyAde^+$, 3-acetylpyridine–adenine dinucleotide; $AmPyAde^+$, 3-aminopyridine–adenine dinucleotide; Cl_2Ind, 2,6-dichloroindophenol; PP_i/EDTA buffer, 50 mM sodium pyrophosphate, 0.5 mM EDTA, pH 8.0.

Enzymes. Lipoamide dehydrogenase, *NADH:lipoamide* oxidoreductase (EC 1.8.1.4); glucose-6-phosphate dehydrogenase, D-glucose-6-phosphate:NADP 1-oxidoreductase (EC 1.1.1.49); xanthine oxidase, xanthine:oxygen oxidoreductase (EC 1.1.3.22)



Scheme 1. Proposed reaction intermediates for lipoamide dehydrogenase. The arrows indicate charge-transfer interaction and are drawn from donor to acceptor.

Lipoamide dehydrogenase is the flavoprotein component of the pyruvate, oxoglutarate and branched-chain-oxoacid dehydrogenase complexes [1, 2]. In the physiological direction, it catalyses the oxidation of a reduced lipoyl group that is covalently attached to the core protein of these complexes by NAD^+. This lipoyl group can be replaced with free lipoamide. Lipoamide dehydrogenase belongs to the family of dimeric flavoenzymes that contain a redox-active disulfide bridge participating in catalysis [1]. The enzyme is composed of two identical subunits with the two active sites built by contacts between the subunits. Other members of this family are glutathione reductase [1], mercuric ion reductase [3], trypanothione reductase [4] and thioredoxin reductase [5].

Extensive studies on lipoamide dehydrogenase have been performed on the enzymes as isolated from pig heart, rat liver and *Escherichia coli* [6–14]. All three lipoamide dehydrogenases were shown to act according to a ping-pong mechanism in the physiological reaction [6, 8, 14]. During catalysis, the enzyme shuttles between the oxidized (E_{ox}) and the two-electron-reduced (EH_2) state [15]. Inhibition due to over-reduction to the four-electron reduced state (EH_4) is most severe in *E. coli* enzyme and only slight in pig heart enzyme [7, 12, 14, 15]. The nature of the dimeric structure indicates that communication between the active sites seems possible. Evidence for positive and negative cooperativity in this family of dimeric enzymes has been reported [8, 14, 16–19].

Detailed studies on pig heart enzyme comprising both steady-state and rapid-reaction kinetics have led to the proposal of a reaction mechanism involving a base, histidine, in the active site that becomes protonated during catalysis [9, 20, 21]. Recently, conclusive evidence for the role of the histidine was obtained by site directed mutagenesis studies on glutathione reductase [22, 23], *E. coli* [24] and *Azotobacter vinelandii* lipoamide dehydrogenase [25]. Scheme 1 depicts proposed and identified reaction intermediates [7, 9–11, 15, 17, 26–30]. Catalysis proceeds around the upper cycle, species **1**–**7**. The lower cycle shows species involved in four-electron reduction, species **8**–**11**.

In a previous paper the rationale for the selection of amino acids to be mutated was outlined and the spectral properties of the wild-type and mutated enzymes were reported [26]. This paper deals with the kinetic properties and identification of several of the intermediate species as shown in Scheme 1 of *A. vinelandii* lipoamide dehydrogenase and mutated enzymes.

MATERIALS AND METHODS

General

Construction of the mutated enzymes, isolation and treatment of the enzymes were performed as described [26]. NAD^+ (grade I) NADH (grade I), $NADP^+$, xanthine, xanthine oxidase (from cow milk), glucose 6-phosphate and glucose-6-phosphate dehydrogenase (from yeast, analytical grade) were from Boehringer. NAD^+ analogs, 6,8-thioctic acid amide ($LipS_2$), dichloroindophenol (Cl_2Ind), 5,5′-dithiobis(2-nitrobenzoate) (Nbs_2) and biological buffers were obtained from Sigma Inc. All other chemicals used were of the highest purity available. NAD^+, acetylpyridine–adenine dinucleotide ($AcPyAde^+$), NADH, reduced $LipS_2$ [$Lip(SH)_2$] and enzyme concentrations were determined as described previously [26]. The thio-NAD^+ concentration was determined as described for $AcPyAde^+$.

Steady-state kinetics

All activity measurements were performed on a Zeiss M4 QIII spectrophotometer at 25°C. The forward reaction

$Lip(SH)_2/NAD^+$ was determined routinely in 50 mM sodium pyrophospate, 0.5 mM EDTA pH 8.0 (PP_i/EDTA buffer). In a standard assay, 1.0 mM NAD^+ and 1.0 mM $Lip(SH)_2$ were used and the formation of NADH was monitored at 340 nm. Steady-state kinetics of the forward reaction of the wild-type enzyme were studied as follows. To a temperature equilibrated cuvette containing 950 µl PP_i buffer, 20 µl of each of the substrates was added, followed by the addition of 10 µl enzyme at the proper dilution. Substrate concentration combinations were chosen such as to avoid errors due to inactivation of enzyme in the time course of the experiment. Therefore the experiment was started with the lowest concentration of substrate A, where substrate B was varied, followed by the highest concentration of substrate A with B varied. Next the second lowest concentration of A was used followed by the second highest concentration of A and so on. Triplicate series were measured.

pH-dependent studies were performed in buffers containing 0.5 mM EDTA and adjusted to ionic strength 150 mM with KCl. Buffers used: pH 5.5–6.0–6.5, 100 mM Mes; pH 7.0–7.5–7.8, 100 mM Hepes; pH 8.0–8.6, 100 mM Hepps; pH 9.0–9.3 100 mM Ches. Data were analyzed according to a ping-pong mechanism.

The reverse reaction, $NADH/LipS_2$, was determined as described [31]. The NADH-dependent reduction of Cl_2Ind (diaphorase activity) was measured in PP_i/EDTA buffer ($\varepsilon_{600\ nm}$ for Cl_2Ind = 22900 M^{-1} cm^{-1} at pH 8.0) by following the decrease of Cl_2Ind at 600 nm. The transhydrogenase reaction NADH/thio-NAD^+ was performed according to [32].

Rapid-reaction kinetics

Rapid-reaction kinetics were carried out using a temperature-controlled single-wavelength stopped-flow spectrophotometer, type SF-51, from High Tech Scientific Inc. with 1.3 ms dead time. The instrument was interfaced to an IBM computer for data acquisition and analysis. Data were analyzed with a program from High Tech Scientific Inc. Rapid-scan experiments were performed with a stopped-flow instrument having a 2-cm light-path cell and a dead time of 3 ms, and using a Tracor Northern diode array spectrophotometer as the detector (scan time 5.42 ms). The detector was interfaced to a Tracor Northern computer for data acquisition and analysis. All experiments were performed anaerobically at 21.0°C in 100 mM Good buffers containing 0.5 mM EDTA adjusted to ionic strength 150 mM with KCl. Enzyme concentrations after mixing were 26.7 µM. Generation of two-electron-reduced enzyme (EH_2) was accomplished by reduction with a small excess of sodium borohydride.

Rate constants at infinite substrate concentration and K_d values were determined from non-linear fitting of apparent rate constants obtained at, at least, five different substrate concentrations. Apparent rate constants represent the average of minimally four shots. In order to achieve pseudo-first-order conditions the lowest substrate concentrations used were five times as high as the enzyme concentration. Since only the L-enantiomer of $Lip(SH)_2$ reacts rapidly [6, 9], substrate concentrations were adjusted to those of the L-enantiomer. $Lip(SH)_2$ and $LipS_2$ were dissolved in buffer/ethanol to yield a final ethanol content of 5% in the mixing chamber.

Simulation of rapid-reaction kinetics was performed using the program KINSIM [33] run on a VAX/VMS minicomputer.

Redox potential determinations

Visible absorption spectra were recorded on a temperature-controlled Aminco DW2000 spectrophotometer at 25°C. Reductions were carried out in anaerobic cuvettes in 50 mM potassium phosphate pH 7.0, 0.5 mM EDTA. Redox potentials were determined in three different ways. Method one was essentially as described for pig heart enzyme [20]. Method two, the xanthine/xanthine oxidase method, was recently described by Massey [34] and was applied without modification. The co-titrants used, 30 µM in concentration, were safranine T, phenosafranine and benzylviologen. Method three was a modification of method two. Instead of using xanthine/xanthine oxidase as an electron generating system, NADPH was generated from $NADP^+$ (75 µM) using glucose-6-phosphate dehydrogenase (0.2–04 mU) and glucose 6-phospate (400 µM). NADPH proved useful as a reductant for lipoamide dehydrogenase since quantitative reduction could be achieved yielding spectra identical to reduction by $Lip(SH)_2$. Furthermore $NADP^+$ did not give rise to the typical long-wavelength absorbance characteristic for $FADH^-/NAD^+$ charge-transfer interaction, indicating that no efficient binding occurs. For method one, the titrant was added using a gas-tight Hamilton syringe equipped with a dispenser. Spectra were recorded until changes were complete. For methods two and three the reaction was started by the addition of enzyme (xanthine oxidase or glucose-6-phosphate dehydrogenase) after anaerobiosis was established. In order to achieve equilibrium the amount of enzyme was chosen such as to complete reduction in a time span of 6–9 h. The amount of enzyme was halved to check whether equilibrium was established [34].

RESULTS

Steady-state kinetics

Wild-type lipoamide dehydrogenase

Under nonsaturating conditions, the optimum pH of the reaction $Lip(SH)_2/NAD^+$ is 8.0, in PP_i/EDTA buffer as found for all other lipoamide dehydrogenases [6–8] Also the reaction $Lip(SH)_2/AcPyAde^+$ shows an optimum at pH 8.0. In Fig. 1 the double-reciprocal plots of the forward reaction $Lip(SH)_2/NAD^+$ are presented. When the concentrations of $Lip(SH)_2$ and NAD^+ or $AcPyAde^+$ are varied in fixed ratios a straight line is obtained in a double-reciprocal plot (not shown). These results clearly demonstrate that *A. vinelandii* lipoamide dehydrogenase, like all other lipoamide dehydrogenases studied so far, functions according to a ping-pong mechanism.

Parameters determined from steady-state kinetic analysis of the wild-type and various mutated forms of lipoamide dehydrogenase are given in Table 1. A Hill plot constructed from the data for the wild-type enzyme reveals a Hill coefficient of 1 with NAD^+ as a substrate excluding cooperativity at the EH_2 level.

To explore the effect of the pH on the activity, steady-state kinetics of wild-type enzyme was studied at different pH values (see Fig. 2A and C) using NAD^+ and $AcPyAde^+$ as electron acceptors. Unexpectedly, strong product inhibition is observed for wild-type enzyme using NAD^+ at high pH values. This will be discussed below. As a result the K_m and V values determined at pH values above pH 8.0 are not reliable and are therefore not included in Fig. 2A. This inhibition is not observed with $AcPyAde^+$ as acceptor.

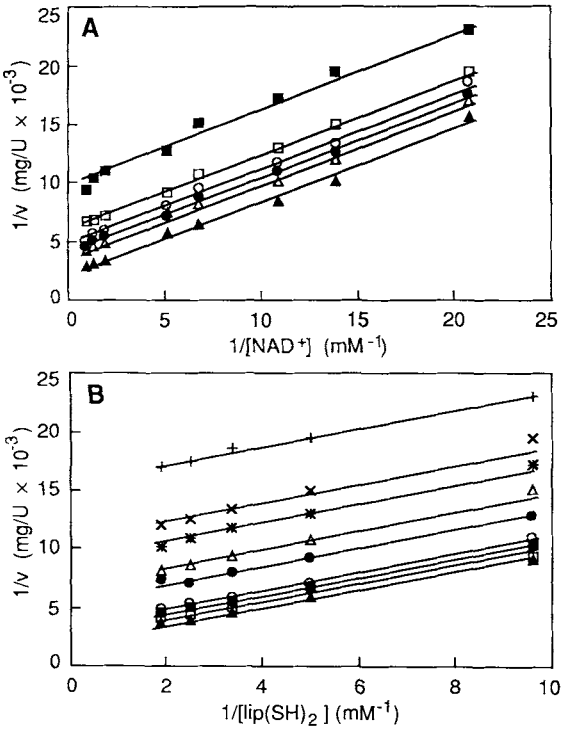


Fig. 1. Steady-state kinetics of the physiological reaction of *A. vinelandii* wild-type lipoamide dehydrogenase at the optimum pH 8.0. The temperature was 25°C; for other details see Materials and Methods. (A) Lineweaver-Burk plot of the reaction $Lip(SH)_2/NAD^+$ with $[NAD^+]$ varied and $[Lip(SH)_2]$ fixed at: (■) 104 μM, (□) 200 μM, (○) 295 μM, (●) 397 μM, (△) 518 μM; (▲) extrapolated velocities at infinite $[Lip(SH)_2]$. (B) As (A) with $[Lip(SH)_2]$ varied and $[NAD^+]$ fixed at: (+) 48 μM, (×) 72 μM, (*) 92 μM, (△) 147 μM, (●) 194 μM, (○) 511 μM, (■) 752 μM, (□) 1044 μM; (▲) extrapolated velocities at infinite $[NAD^+]$.

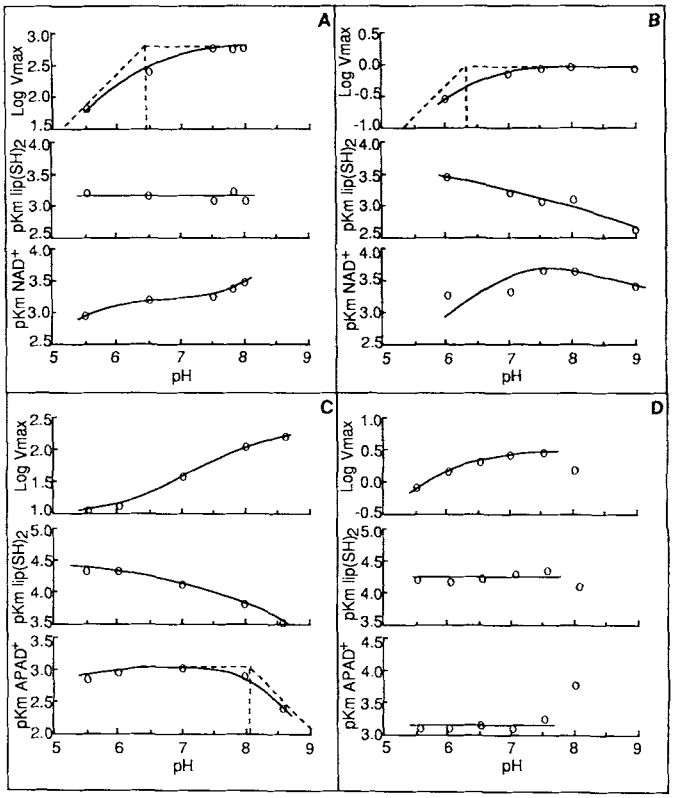


Fig. 2. pH dependence of the steady-state kinetics in the physiological reaction of *A. vinelandii* wild-type lipoamide dehydrogenase and mutated enzymes His450 → Ser and Glu455 → Asp. Conditions are described in Materials and Methods. (A) Wild-type enzyme using NAD^+ as electron acceptor; (B) enzyme His450 → Ser using NAD^+ as electron acceptor; (C) wild-type enzyme using $AcPyAde^+$ as electron acceptor; (D) enzyme Glu455 → Asp using $AcPyAde^+$ as electron acceptor.

Table 1. Steady-state kinetic parameters of *A. vinelandii* wild-type and mutated enzymes. The parameters were determined at pH 8.0, the pH optimum for each enzyme, except for enzyme Glu455 → Asp where the optimum was pH 7.0. nd signifies that kinetics are not studied due to strong product inhibition. A question mark (?) for enzyme Pro451 → Ala signifies that the parameters can not be determined due to very transient activity (see text also). The concentration of $Lip(SH)_2$ is for the DL-mixture.

Enzyme	$Lip(SH)_2/NAD^+$			$Lip(SH)_2/AcPyAde^+$			$NADH/Cl_2Ind$	
	k_{cat}	K_m $Lip(SH)_2$	K_m NAD^+	k_{cat}	K_m $Lip(SH)_2$	K_m $AcPyAde^+$	k_{cat}	K_m NADH
	s^{-1}	μM		s^{-1}	μM		s^{-1}	μM
Wild-type	420	390	325	100	140	1200	170	180
*His*450→Ser	1.6	1950	300	—	—	—	135	140
His450→Phe	0.0	—	—	—	—	—	170	150
His450→Tyr	0.0	—	—	—	—	—	170	200
Glu455→Asp	nd	—	—	2.4	30	640	160	50
Glu455→Gln	nd	—	—	1.9	240	1750	200	120
Pro451→Ala	?	—	—	—	—	—	?	—

From the log(V) plot in Fig. 2A, a pK_a value of 6.4 is obtained for the rate-limiting intermediate of wild-type enzyme. The invariance of pK_m $[Lip(SH)_2]$ and the change in the pK_m $[NAD^+]$ around pH 6.4 suggests that protonation of the $EH_2 \cdot NAD^+$ complex is rate-limiting. This is supported by rapid-reaction kinetics which show that at pH 8.0 and 6.8 the reoxidation by NAD^+ is rate-limiting (see below).

Fig. 2C shows that V for $AcPyAde^+$ reduction by wild-type enzyme does not reach a maximum in the experimental pH interval. Therefore no pK_a can be assigned to a rate-limiting intermediate. From the pK_m $[AcPyAde^+]$ plot a pK_a of 8.1 is determined representing a deprotonation of EH_2 [35]. This pK_a value may reflect deprotonation of the histidine.

His450-mutated enzymes

With enzymes His450 → Phe and His450 → Tyr virtually no activity is observed in either direction. Spectral studies

showed that these enzymes still can be reduced by $Lip(SH)_2$ though very slowly. It is therefore concluded that the rather bulky aromatic residues impose structural constraints on the enzyme that make it very difficult for $Lip(SH)_2$ to interact effectively with the disulfide to exchange reducing equivalents. With enzyme His450 → Ser almost no activity was observed in the reverse reaction, reduction of $LipS_2$, monitored by the oxidation of NADH in the presence of NAD^+. About 0.5% of the wild-type activity is found in the forward reaction, $Lip(SH)_2/NAD^+$, at the optimum pH 8.0 A kinetic analysis as described for wild-type enzyme demonstrates that enzyme His450 → Ser also functions according to a ping-pong mechanism.

Assuming His450 to abstract a proton from the substrate $Lip(SH)_2$ as proposed [9], one might expect the activity of enzyme His450 → Ser would increase at elevated pH values, where $Lip(SH)_2$ (pK_{a1} = 9.35, free in solution [9]) becomes deprotonated. However V remains constant between pH 8.0 and pH 9.0. In the pH-dependent studies no product inhibition by NADH is found in the experimental pH interval. From the log(V) plot in Fig. 2B, a pK_a value of 6.3 is obtained for the deprotonation of the rate-limiting intermediate in enzyme His450 → Ser.

Glu455-mutated enzymes

With enzyme Glu455 → Asp very strong product (NADH) inhibition is observed in the reaction $Lip(SH)_2/NAD^+$. The results indicate that this enzyme is inhibited by NADH in a 'dead-end' manner. Addition of excess NAD^+ after inhibition is complete does not restore activity. Revived NADH production is only observed after the addition of an extra aliquot of enzyme. This extra enzyme portion becomes inhibited approximately as fast as the initial enzyme portion. No detailed studies were carried out to investigate this phenomenon. However the results indicate that the inhibition is more dependent on time than on the NADH concentration. In view of an increase of the dissociation constant for the monomer dimer equilibrium of the wild-type enzyme upon reduction to the EH_4 level [31], it is tempting to speculate that the irreversibility of the inhibition is caused by monomerization.

The reverse reaction as well as the transhydrogenase reaction NADH/thio-NAD^+ are not detectable. When an electron acceptor with higher midpoint potential than NAD^+ or thio-NAD^+ is used, *in casu* of $AcPyAde^+$ in the forward reaction, and Cl_2Ind in the diaphorase reaction, quantitative reduction of the acceptors is observed in the pH interval studied (pH 5.5 – 9.0) (E'_m NAD^+/NADH = -320 mV [36], E'_m thioNAD^+/thioNADH = −283 mV [36], E'_m $AcPyAde^+$/AcPyAdeH = −258 mV [36] and E'_m Cl_2Ind/Cl_2IndH_2 = +217 mV [37], pH 7.0). With $AcPyAde^+$ 'dead-end' inhibition is observed above pH 7.5.

Enzyme Glu455 → Gln differs in catalytic behavior from enzyme Glu455 → Asp. Here a somewhat less strong inhibitory effect of NADH is found. With $AcPyAde^+$ as acceptor no inhibition is observed.

Under non-saturating fixed substrate concentrations the optimum for the $AcPyAde^+$ reduction is at pH 8.0 for enzyme Glu455 → Gln and is lowered to pH 7.0 – 7.5 for enzyme Glu455 → Asp. Both Glu455-mutated enzymes function according to a ping-pong mechanism.

For both Glu455-mutated enzymes the steady-state kinetics of the reaction $Lip(SH)_2/AcPyAde^+$ were studied as a function of pH. Enzyme Glu455 → Asp shows, as mentioned above, 'dead-end' inhibition with $AcPyAde^+$. This hampers the determination of the kinetic constants above pH 7.5. Except for a change in V, the kinetic parameters of enzyme Glu455 → Asp do not change significantly in the experimental pH range (pH ≤ 7.5, Fig. 2 D). The pH dependence of steady-state kinetics of enzyme Glu455 → Gln is quite different from enzyme Glu455 → Asp (results not shown). V changes only slightly over the experimental pH range (pH 5.5 – 9.0) showing an optimum at pH 8.0. The K_m for $Lip(SH)_2$ increases at lower pH but K_m $AcPyAde^+$ remains almost constant as in enzyme Glu455 → Asp. No pK_a values are determinable for this enzyme.

Rapid-reaction kinetics

In order to obtain a more detailed insight into the separate half reactions, the reductive reactions with $Lip(SH)_2$ and NADH and the oxidative reactions with NAD^+ and $LipS_2$ were studied in the stopped-flow spectrophotometer.

Wild-type lipoamide dehydrogenase

At pH 7.0 and 8.0 the reduction of E_{ox} by $Lip(SH)_2$ and reoxidation of EH_2 by NAD^+ and $LipS_2$ as monitored at 450 nm and 530 nm can essentially be described by a single-exponential function. In Table 2 the extrapolated rate constants at infinite substrate concentration and corresponding K_d values are presented. Ping-pong enzymes obey the following equations:

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E' + P \quad (1)$$

$$E' + B \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E'B \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E + Q \quad (2)$$

for which the steady-state relation

$$k_{cat} = \frac{k_3 \times k_7}{k_3 + k_7} \quad (3)$$

was derived [38]. Here k_3 and k_7 are the first-order rate constants of the reaction of the first and second substrate respectively (k_a of the appropriate substrate in Table 2). A k_{cat} was calculated as 670 s^{-1} at pH 8.0 for the reaction $Lip(SH)_2/NAD^+$ with data from Table 2 and Eqn (3). This value agrees reasonably well with k_{cat} calculated from steady-state kinetics: 420 s^{-1}. Comparison of k_3 and k_7 demonstrates that, at pH 8.0, the reductive reaction by $Lip(SH)_2$ is not rate-limiting as found for the pig heart enzyme [6, 10].

The reductive reaction with NADH is extremely fast and is almost complete in the dead time of the stopped-flow instrument. Rapid scan spectra (dead time spectrum 5.42 ms after mixing) and a compilation of relative absorbance changes at 453, 530 and 700 nm collected with the single-wavelength instrument are shown in Fig. 3. The absorption at 700 nm is a monitor for the charge-transfer complex between the reduced flavin ($FADH^-$) and NAD^+ (species 6 in Scheme 1, see [26] for the spectrum of this species). Fig. 3A shows that approximately half of the total absorbance change at 453 nm takes place in 10 ms, but Fig. 3B shows that only 20% of the total absorbance change takes place in 10 ms. The fact that the 700-nm absorbance is already up at 0.5 ms indicates that a very fast phase, involving a significant decrease in absorbance at 453 nm, was largely missed. These initial changes are logically attributed to the formation of the $FADH^-/NAD^+$ charge-transfer complex. The 530-nm absorbance increases over the first 5 ms and indicates that the electrons (formally

Table 2. Rate constants and dissociation constants of *A. vinelandii* wild-type and mutated enzymes. For NADH reduction the rate constant of reduction of the FAD is shown. A question mark (?) signifies that the constants could not be determined due to the small absorbance changes associated at low substrate concentration (see text). K_d values for $Lip(SH)_2$ are expressed for the L-enantiomer. Rate constants k_a and k_b are extrapolated to infinite [substrate]; the faster rate constant is k_a while the slower rate constant is k_b. The wavelength at which data were obtained is indicated in the last column.

Enzyme	Reduction level	Substrate	pH	k_a	$K_{d(a)}$	k_b	$K_{d(b)}$	Wavelength
				s^{-1}	mM	s^{-1}	mM	nm
Wild-type	E_{ox}	$Lip(SH)_2$	8.0	2000	0.9			450, 530
	E_{ox}	$Lip(SH)_2$	7.0	1000	0.7			530
	E_{ox}	NADH	8.0	>3000				450, 530, 700
	EH_2	$LipS_2$	8.0	400	0.5			450, 530
	EH_2	NAD^+	8.0	1000	0.37			450, 530
	EH_2	NAD^+	7.0	760	0.47			450, 530
His450→Ser	E_{ox}	$Lip(SH)_2$	9.3	1.9	5.0			450, 530
	E_{ox}	$Lip(SH)_2$	8.6	1.5	4.3			530
	E_{ox}	$Lip(SH)_2$	7.8	1.3	4.7			530
	E_{ox}	$Lip(SH)_2$	7.3	1.0	3.7			530
	E_{ox}	$Lip(SH)_2$	6.6	0.8	3.5			530
	E_{ox}	NADH	8.0	>3000				450, 530, 700
	EH_2	$LipS_2$	8.0	<0.005				530
	EH_2	NAD^+	8.0	4.0	0.33			530
	EH_2	NAD^+	6.8	4.4	0.27			530
Glu455→Asp	E_{ox}	$Lip(SH)_2$	8.0	?	?	1000	4.7	450, 530
	E_{ox}	$Lip(SH)_2$	7.0	?	?	600	4.0	530
	E_{ox}	$Lip(SH)_2$	5.9	230	5.4			530
	E_{ox}	NADH	8.0	>3000				
	EH_2	$LipS_2$	8.0	?	?	12	0.55	450, 530
Glu455→Gln	E_{ox}	$Lip(SH)_2$	8.6	250	2.6	60	2.5	530
	E_{ox}	$Lip(SH)_2$	7.9	?	?	60	4.2	530
	E_{ox}	$Lip(SH)_2$	7.3	?	?	35	3.6	530
	E_{ox}	$Lip(SH)_2$	5.9	44	4.8			530
	E_{ox}	NADH	8.0	>3000				450, 530, 700
	EH_2	$LipS_2$	8.0	<0.05				530
	EH_2	NAD^+	8.0	58	0.17	14	0.30	530

two electrons and a proton; a hydride equivalent) are passed over from the flavin to the disulfide (species **6** to **4** or **3** in Scheme 1), an effect that is more pronounced in the His450→Ser enzyme (see below). Changes occurring after 5 ms are most likely due to further reduction by the excess of NADH.

His450-mutated enzymes

The reductive reaction of E_{ox} by $Lip(SH)_2$ is slow (His450→Ser) to extremely slow (His450→Phe and His450→Tyr) upon mutation of the active-site base. No rate constants were determined for enzymes His450→Phe and His450→Tyr. Even at the highest possible substrate concentration full reduction to EH_2 lasted 250 s, yielding an apparent first order rate constant of 25×10^{-3} s^{-1}. For enzyme His450→Ser the reduction by $Lip(SH)_2$ is monophasic preceded by a lag phase (< 300 ms). The reoxidation of EH_2 by NAD^+ is also monophasic but the lag phase is not observed in these traces. The rate constants and K_d values as determined for enzyme His450→Ser are presented in Table 2. The rate constants and K_d values for the substrates L-$Lip(SH)_2$ and L-$LipS_2$ should be regarded as approximations since the determined K_d values are approximately 3–4 times higher than the maximal experimental concentration of L-$Lip(SH)_2$ or L-$LipS_2$ due to solubility limitations.

For the forward overall reaction of enzyme His450→Ser from Eqn. (3) a k_{cat} of 1.0 s^{-1} is calculated that agrees reasonably well with k_{cat} = 1.6 s^{-1} as obtained from steady-state kinetics. Contrary to the wild-type enzyme however, for enzyme His450→Ser the reductive reaction is rate-limiting at both pH 7.0 and 8.0.

The rate constant for the reductive reaction by $Lip(SH)_2$ increases only slightly with pH and the traces remain monoexponential up to pH 9.3. The K_d for $Lip(SH)_2$ increases with pH suggesting that the substrate binds to the mutated enzyme in its protonated state. The reoxidation reaction of His450→Ser EH_2 by $LipS_2$ is barely detectable at pH 8.0, indicating that the reactivity of the substrate is very poor or the affinity of the enzyme for this substrate is very low.

The reoxidation of EH_2 by NAD^+ proceeds faster in this enzyme than the reduction of E_{ox} by $Lip(SH)_2$ (Table 2) although the reaction is also extremely slow when compared to wild-type enzyme.

A possible mechanism of electron transfer from the nascent thiols to the flavin involves the transient formation of a C4a adduct between the charge-transfer thiolate and the flavin [17, 27, 28], species **5** in Scheme 1. This flavin C4a adduct species exhibits characteristic absorbance at 380 nm. Therefore the reoxidation of His450→Ser EH_2 with NAD^+ was monitored at this wavelength. No absorbance changes compatible with the formation of a flavin C4a adduct species were however detected.

The kinetics of the reduction of enzyme His450→Ser by NADH are compatible with a very fast reduction of the flavin

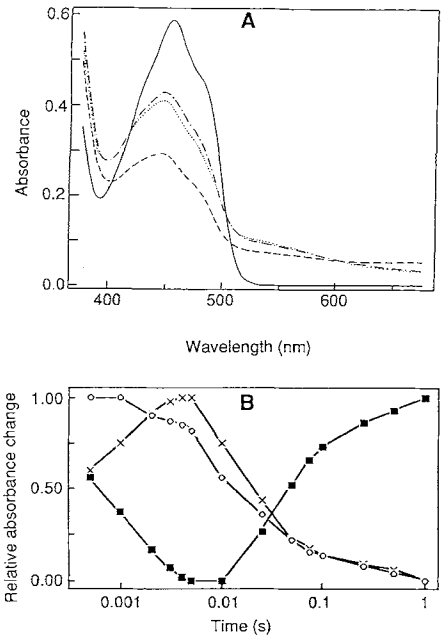


Fig. 3. Spectral changes of *A. vinelandii* wild-type lipoamide dehydrogenase upon reduction by NADH in the rapid-reaction instruments. (A) Reduction of wild-type enzyme by 5 mol NADH/mol at pH 7.8, recorded with the rapid-scan instrument. (——) E_{ox}; (— · —) dead time (5.42 ms after mixing); (· · · · · ·) 10.84 ms after mixing; (— — —) 542 ms after mixing. (B) Compilation of relative absorbance changes during reduction of wild-type enzyme by 10 mol NADH/mol in the single-wavelength stopped-flow instrument. (◇) Absorbance changes at 453 nm; (×) absorbance changes at 530 nm; (■) absorbance changes at 700 nm.

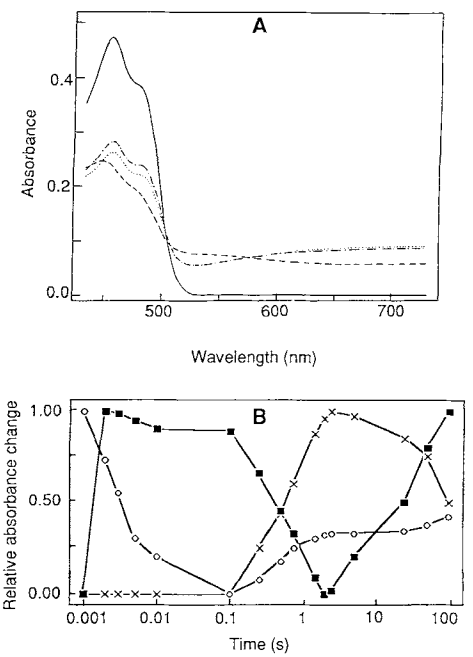


Fig. 4. Spectral changes of *A. vinelandii* His450 → Ser lipoamide dehydrogenase upon reduction by NADH in the rapid-reaction instruments. (A) Reduction of enzyme His450 → Ser by 10 mol NADH/mol at pH 7.8, recorded with the rapid scan instrument. (——) E_{ox}; (— · —) dead time (5.42 ms after mixing); (· · · · · ·) 10.84 ms after mixing; (— — —) 542 ms after mixing. (B) Compilation of relative absorbance changes during reduction of enzyme His450 → Ser by 10 mol NADH/mol in the single-wavelength stopped-flow instrument. (◇) Absorbance changes at 453 nm; (×) absorbance changes at 530 nm; (■) absorbance changes at 700 nm.

(species **6** in Scheme 1) with subsequent slow transfer of the hydride equivalent to the disulfide (species **4** and **3** in Scheme 1). Fig. 4 shows rapid-scan spectra and a compilation of relative absorbance changes at 453, 530 and 700 nm obtained with the single-wavelength instrument. The profound stabilization of the $FADH^-/NAD^+$ charge-transfer complex by a factor of 1000 is due to impaired transfer of electrons to the disulfide. This intramolecular transfer of a hydride equivalent involves partial regain of absorbance at 453 nm, loss of $FADH^-/NAD^+$ charge-transfer at 700 nm and gain of the thiolate to FAD charge-transfer absorbance at 530 nm. Following reduction of the disulfide, reaction with a second molecule of NADH is very slow (species **3** to **11**). Also in the reductive reaction with NADH no 380-nm absorbance is detected that would indicate the transient stabilization of a flavin C4a adduct species.

Glu455-mutated enzymes

For the Glu455-mutated enzymes the kinetic traces obtained upon reduction of E_{ox} with $Lip(SH)_2$ or reoxidation of EH_2 with either $LipS_2$ or NAD^+ at pH > 6.0 are not mono-exponential as found for the wild-type and His450 → Ser enzymes (Table 2). The introduction of a second exponential demonstrates that the reaction proceeds in at least two phases. In all three reactions studied the biphasic nature becomes more apparent at high substrate concentrations as the amplitude of the fast phase increases. Except for enzyme Glu455 → Gln in the reductive reaction at pH 8.6 and in the reoxidation reaction with NAD^+ at pH 8.0 no rate constant for the fast phase can confidently be determined due to the small absorbance changes causing variability in apparent rate constants determined at low substrate concentration. For enzyme Glu455 → Gln in the reductive half reaction at pH 8.6, the contribution of the amplitude of the fast phase increases from 5% at 250 μM $Lip(SH)_2$ to 30% at 1.7 mM $Lip(SH)_2$ and extrapolates to 50% at infinite $Lip(SH)_2$ concentration suggesting that only half of the enzyme reacts in the fast phase. This will be discussed below. In the oxidative reaction with NAD^+ at pH 8.0, a similar feature is observed. The contribution of the amplitude of the fast phase increases from 25% at $[NAD^+] = K_d$ to 58% at $[NAD^+] = 10$ times K_d. In this reaction no absorbance changes at 380 nm are found that indicate the formation of a C4a adduct species. The oxidative reaction of EH_2 of enzyme Glu455 → Gln with $LipS_2$ at pH 8.0 is very slow and almost beyond detection.

Fig. 5 shows rapid-scan spectra obtained from reoxidation of EH_2 of Glu455 → Gln by 20 mol NAD^+/mol. EH_2 binds NAD^+ rapidly to give a spectrum that closely resembles the absorption spectra obtained from titration of pig heart EH_2 with NAD^+ [10] and titration of pig heart enzyme with NADH in the presence of high $[NAD^+]$ [39]. This spectrum represents a reduced-disulfide/oxidized flavin/NAD^+ intermediate species (species **4** in Scheme 1). Intramolecular transfer of a hydride equivalent from the dithiol to the flavin is biphasic and relatively slow, and results in reduction of most of the flavin despite the presence of a large excess of NAD^+.

The reductive reaction of E_{ox} Glu455 → Gln with 2 mol NADH/mol is shown in Fig. 6. The dead-time spectrum shows that the reduction of the FAD is very fast (species **6** in Scheme 1). The subsequent intramolecular electron transfer

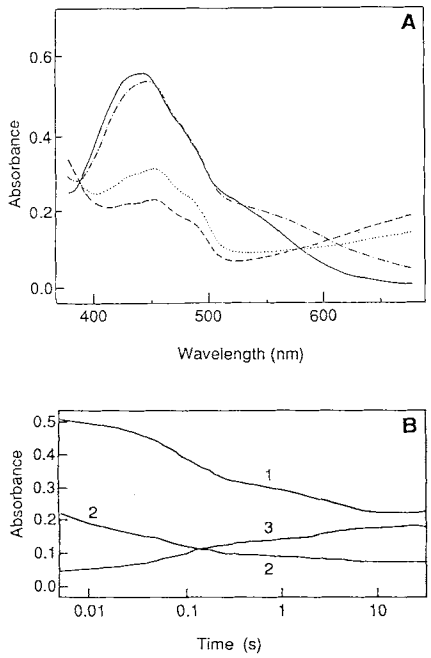


Fig. 5. Spectral changes of two-electron reduced *A. vinelandii* Glu455 → Gln lipoamide dehydrogenase upon oxidation by NAD^+ in the rapid-scan instrument. (A) Oxidation of two-electron-reduced enzyme Glu455 → Gln by 20 mol NAD^+/mol at pH 7.8 in the rapid-scan spectrophotometer. (——) EH_2; (— · —) dead time (5.42 ms after mixing); (·····) 306 ms after mixing; (— — —) 30.6 s after mixing. (B) Time dependence of absorbance changes of A. Curve 1, 457 nm; curve 2, 530 nm; curve 3, 670 nm. EH_2 was generated by reduction with an excess of sodium borohydride, with time for the excess to self destruct.

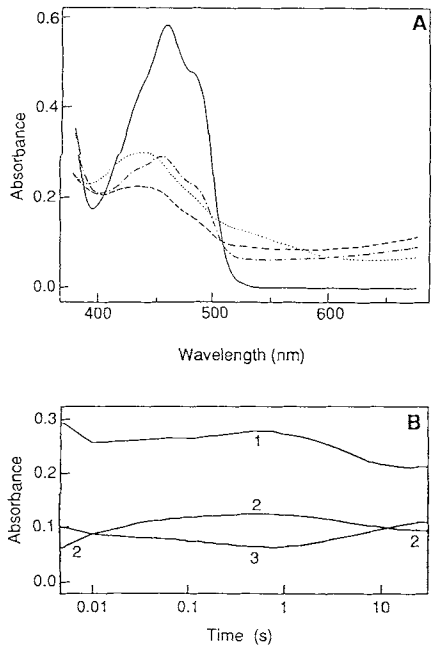


Fig. 6. Spectral changes of *A. vinelandii* Glu455 → Gln lipoamide dehydrogenase upon reduction by NADH in the rapid-scan instrument. (A) Reduction of enzyme Glu455 → Gln by 2 mol NADH/mol at pH 7.8 in the rapid-scan spectrophotometer. (——) E_{ox}; (— · —) dead time (5.42 ms after mixing); (·····) 612 ms after mixing; (— — —) 30.6 s after mixing. (B) Time dependence of absorbance changes of A. Curve 1, 457 nm; curve 2, 530 nm; curve 3, 670 nm.

to the disulfide is again relatively slow. Following reduction of the disulfide, reaction with a second molecule of NADH is slow.

Enzyme Glu455 → Asp differs from Glu455 → Gln in spectral properties during reduction by NADH (Table 2). At all NADH concentrations used, enzyme Glu455 → Asp shows more extensive reduction of the flavin than enzyme Glu455 → Gln and the subsequent reactions are less clear (results not shown).

The results obtained from the rapid reaction studies on the Glu455-mutated enzymes clearly demonstrate that the intramolecular transfer of a hydride equivalent in either direction is slowed down though not as severely as in enzyme His450 → Ser. The underlying mechanism might be the same as for enzyme His450 → Ser and will be discussed below.

Enzyme Pro451 → Ala

The enzyme Pro451 → Ala appears to be essentially inactive in both directions. Spectral studies however demonstrate that this enzyme can be reduced by both $Lip(SH)_2$ and NADH, but rapid over-reduction to the catalytically inactive EH_4 occurs. Stopped-flow studies at 530 and 450 nm show the rapid formation of a transient charge-transfer complex between thiolate and FAD upon reduction with $Lip(SH)_2$ (within 5 s) followed by a slower reduction of the flavin (results not shown). Enzyme Pro451 → Ala also becomes fully reduced by NADH in 10 ms without any detectable changes at 530 nm. The fact that the reduction by NADH is much faster than the over-reduction by $Lip(SH)_2$ indicates that the absence of detectable activity in the physiological reaction is mainly due to the over-reduction by NADH generated after one or a few cycles of turnover.

Redox potentials

Spectral studies have suggested differences in redox potentials between wild-type and mutated enzymes [26]. The different sensitivity of the enzymes toward inhibition by NADH also indicates that differences in redox potentials may exist. Quantitative estimation of the redox potentials as measured by three different methods, however, failed. The main reason for this is the uncertainty as to the exact contribution of the various EH_2 species. During the reduction to EH_2 and subsequently EH_4 no isosbestic points are observed.

Although no exact redox potentials are determinable, the results obtained allow an estimation of redox potentials of the enzymes in relation to each other. The arrangement of the enzymes in order of decreasing redox potentials (E'_m EH_2/EH_4) is: Glu455 → Asp, Glu455 → Gln, wild-type and His450 → Ser. This trend coincides with the sensitivity towards inhibition by NADH.

Several lines of evidence indicate that binding of NAD^+ induces an increase of the redox potential of the FAD [10, 40]. Therefore the course of reduction of wild-type enzyme and enzyme Glu455 → Asp by NADPH in an NADPH-generating system was studied in the presence of 100 μM 3-aminopyridine–adenine dinucleotide ($AmPyAde^+$) [10, 17, 41], a non-reducible NAD^+ analog [K_i for $AmPyAde^+$ with the wild-type enzyme in the reaction $Lip(SH)_2/NAD^+$ is 40 μM]. The formation of NADPH is rate-limiting in both the absence and presence of $AmPyAde^+$ and equal amounts of glucose-6-phosphate dehydrogenase were used. The results are very

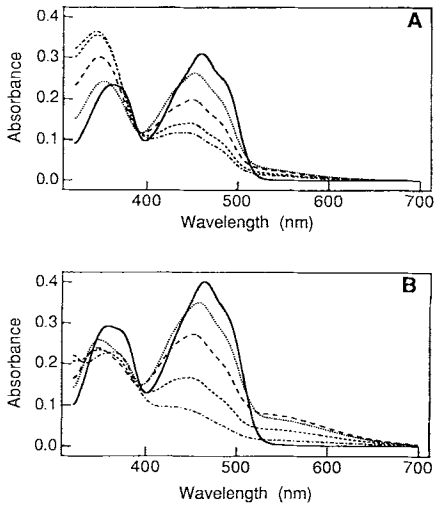


Fig. 7. Spectral changes upon reduction of *A. vinelandii* wild-type enzyme by NADPH in the absence/presence of AmPyAde$^+$. Conditions for reduction of wild-type enzyme by enzymatically formed NADPH are described in Materials and Methods. (A) Reduction of wild-type enzyme by NADPH. Selected spectra are shown. (B) Reduction of wild-type enzyme by NADPH in the presence of 100 μM AmPyAde$^+$. Selected spectra are shown. A correction was made for the contribution of AmPyAde$^+$. The same amount of glucose-6-phosphate dehydrogenase was used in A and B.

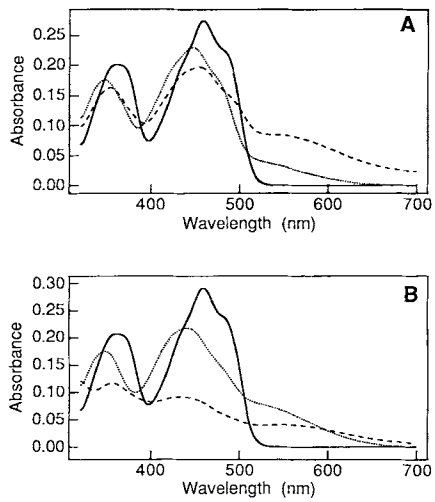


Fig. 8. The effect of addition of AmPyAde$^+$ to EH_2 of *A. vinelandii* lipoamide dehydrogenase. EH_2 was generated by the addition of 5 mol $Lip(SH)_2$/mol enzyme in an anaerobic cuvette at pH 7.0. Spectra shown were recorded after changes were complete. (——), E_{ox}; (·····) EH_2; (— — —) after addition of 200 μM AmPyAde$^+$. (A) Wild-type lipoamide dehydrogenase; (B) Glu455 → Asp lipoamide dehydrogenase.

conclusive (Fig. 7, only wild-type enzyme shown): no NADPH absorbance is observed in the presence of AmPyAde$^+$ until the enzyme is almost completely reduced to EH_4. These results for the first time clearly establish that binding of a pyridine nucleotide (AmPyAde$^+$) indeed raises the EH_2/EH_4 redox potential. Additional support is obtained by the addition of AmPyAde$^+$ to EH_2 of wild-type enzyme and enzyme Glu455 → Asp, generated by the addition of 5 mol $Lip(SH)_2$/mol (Fig. 8). Before addition of AmPyAde$^+$ was started the EH_2 spectra were at equilibrium. Addition of 40 μM AmPyAde$^+$ results in a shift towards longer wavelength and an increase of the charge-transfer absorbance for both enzymes (spectra not shown) similar to the result reported for the pig heart enzyme [10]. Subsequent additions of AmPyAde$^+$ (up to 200 μM) have no effect on the initially obtained spectrum of wild-type enzyme after addition of 40 μM AmPyAde$^+$. However for enzyme Glu455 → Asp a bleaching of the spectrum is observed, demonstrating further reduction to EH_4 by the residual $Lip(SH)_2$. For enzyme Glu455 → Asp it is thus clear that AmPyAde$^+$ effects an increase in the redox potential of the FAD. For wild-type enzyme the further reduction to EH_4 is not observed though a profound enhancement of the 530-nm absorbance occurs which may also be related to a change of the redox potential of the FAD, as was suggested by Matthews and co-workers [10] and Thorpe and Williams [17].

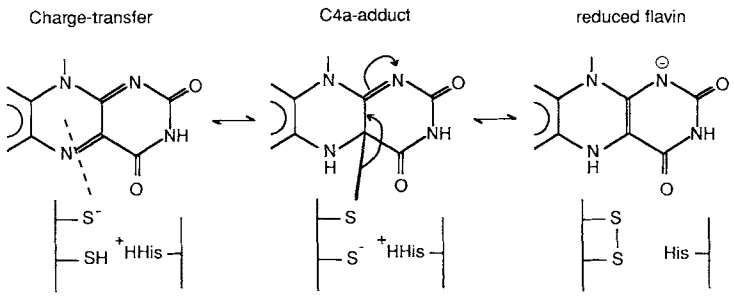
DISCUSSION

The reductive reaction of wild-type and all mutated enzymes with NADH allows the distinction of separate steps, a feature that is most prominent in enzyme His450 → Ser where the rate of reduction of the disulfide by NADH is slowed by more than 1000 fold. The binding of NADH and subsequent reduction of the FAD is in all cases very fast yielding the FlH^- · NAD^+ species as an intermediate, species **6** in Scheme 1. The subsequent intramolecular transfer of a hydride equivalent leading to reduction of the disulfide and reoxidation of the FAD (species **4**) is strongly affected by the mutations. Also, the forward reaction (species **4** to **6**) is slowed by the mutations, more than 200 fold for enzyme His450 → Ser.

Evidence for the formation of the proposed flavin C4a adduct intermediate (species **5**) as shown in Scheme 2 is not obtained under the experimental conditions applied. A plausible explanation for this is that the rate of formation of the presumed C4a adduct is slow in either direction when compared to its breakdown. Leichus and Blanchard [42] showed recently that in the forward reaction of the pig heart enzyme the formation of the proposed species **5** is most likely the solvent isotopically sensitive transfer step under conditions of saturating [$Lip(SH)_2$] and variable [NAD^+]. This is in agreement with our suggestion above, that in the enzymes having either the base, His450, or its charge-relay partner, Glu455, altered, formation of the C4a adduct is slow relative to its breakdown.

However, in the reverse reaction of pig heart enzyme the formation of species **4** is most likely solvent isotopically sensitive under conditions of saturating [$LipS_2$] and variable [NADH] [41]. This is in contrast with the results obtained for the mutated enzymes where, again, no C4a adduct is observed. Two possible reasons for this can be suggested. First, the decay of the C4a adduct might be faster than its formation. Second, in the absence of a base, the transfer of a hydride equivalent may become concerted. Upon opening of the disulfide to form the C4a adduct species, a negative charge develops on the interchange sulfur. It is quite posible that a protonated histidine, the base, is necessary to stabilize this negative charge, and therefore, the second possibility is probably favored.

The reoxidation reaction of EH_2 of enzyme His450 → Ser by NAD^+ is not significantly enhanced at pH 7.0 when compared to pH 8.0. This is unexpected in the light of the following. This mutated enzyme stabilizes thiolate to FAD charge-transfer. Therefore, reduction by $Lip(SH)_2$ requires the loss of one proton to reach a form analogous to species **3** without



Scheme 2. Proposed formation of the intermediate C4a adduct species. Adapted from [17].

the base, EH^-. A pK_a was detected at 7.5 for the His450 → Ser enzyme and interpreted as an EH^- to E^{2-} deprotonation [26]. This deprotonation should disfavor the reduction of NAD^+. The almost complete absence of any reactivity at the EH_2 level of enzyme His450 → Ser with $LipS_2$ at pH 8.0 agrees with the observation for pig heart enzyme lacking reactivity of EH^- with $LipS_2$ [9], and our conclusion is that the main electronic species of two-electron reduced enzyme His450 → Ser is E^{2-} [26].

The reductive reaction of enzyme His450 → Ser by $Lip(SH)_2$ is very slow and fully compatible with the mechanism proposed [1, 9]. The observed short lag phase is in agreement with a slow formation of the proposed mixed disulfide species (assuming that this species has no absorbance) as the catalyst, histidine, is missing. Simulation of the reaction shows that traces compatible with observed traces are only obtained when the breakdown of the mixed disulfide occurs 10-fold faster than its formation. From this we conclude that the rate-limiting step in the reduction of enzyme His450 → Ser by $Lip(SH)_2$ is the formation of the mixed disulfide intermediate.

The Glu455-mutated enzymes are quite different in their rapid reaction kinetics from wild-type enzyme. The data indicate that, like the His450 → Ser enzyme, intramolecular electron transfer between FAD/$FADH^-$ and the disulfide/dithiol is severely impaired. Thus, any alteration of the His-Glu diad affects not only deprotonation of the dithiol substrate, but also communication between the enzyme's two redox-active couples. In addition, the Glu455-mutated enzymes differ from His450 → Ser enzyme in several details of their rapid-reaction kinetics. Both enzymes Glu455 → Asp and Glu455 → Gln show clear biphasic kinetics during reduction of E_{ox} with $Lip(SH)_2$ or reoxidation of EH_2 with either $LipS_2$ or NAD^+. An interesting feature of this biphasic behavior is the fact that the amplitude of the fast reaction increases with substrate concentration and that it reaches about 50% of the total amplitude change. This indicates that only one of the active sites of enzyme Glu455 → Gln is reduced at the rapid rate. Moreover, the K_d values calculated for the fast phase are equal to [enzyme Glu455 → Gln with $Lip(SH)_2$ at pH 8.6] or lower than (enzyme Glu455 → Gln EH_2 with NAD^+ at pH 8.0) those calculated for the slower amplitude change. These results strongly indicate that cooperativity between the two active sites of the enzyme occurs.

The results (Fig. 7) obtained for the reduction of wild-type enzyme by NADPH in the presence of $AmPyAde^+$ support cooperativity between the subunits. Since lipoamide dehydrogenase possesses only one NAD^+ binding site/active site, based on the extensive structural homology between glutathione reductase and lipoamide dehydrogenase from *Pseudomonas putida* [43], binding of both $AmPyAde^+$ and NADPH to one active site simultaneously can be excluded.

Alteration of Glu455, the charge-relay residue in the base diad, effects profound changes in catalysis. The $Lip(SH)_2$/NAD^+ activity is unmeasurable due to over-reduction by the product NADH. The $Lip(SH)_2$/$AcPyAde^+$ activity is about 2% of wild-type. Glu455 alters the acid-base properties of His450 [26] and positions the imidazole ring presenting its N3 toward the redox-active disulfide. Both mutations can be considered conservative, Glu455 → Gln and Glu455 → Asp. Indeed, the almost normal rate of reduction of Glu455 → Asp enzyme by $Lip(SH)_2$ demonstrates that the aspartate can mimick the catalytic effect of the glutamate in the enzyme-substrate complex. Neither the aspartate nor the glutamine residues are able, however, to accomplish the function of the base diad in the intramolecular transfer of electrons between FAD/$FADH^-$ and the disulfide/dithiol. In contrast to the His450 → Ser enzyme where the problem appears to be largely kinetic, the effect with enzymes Glu455 → Gln and Glu455 → Asp seems to be both kinetic and thermodynamic. Thus, the extent of reoxidation of two-electron-reduced enzyme Glu455 → Gln by NAD^+ is very small (Fig. 5). The binding of NAD^+ raises the redox potential of FAD relative to that of the dithiol. Electrons pass, albeit slowly, to the FAD but the resulting $FADH^-$ is not reoxidized.

Inhibition of *A. vinelandii* wild-type enzyme by NADH above pH 8.0 is not observed in pig heart enzyme while for *E. coli* enzyme this inhibition is observed at all pH values studied. For *E. coli* enzyme it was shown that the inhibition is caused by over-reduction of the enzyme from EH_2 to EH_4 by NADH and that there is a direct relation between the inhibition and the redox potential of the EH_2/EH_4 couple [12]. Like the *E. coli* enzyme [7], inhibition of the *A. vinelandii* enzyme could be circumvented in kinetic studies by using $AcPyAde^+$ as an electron acceptor which has a 70 mV higher midpoint potential than NAD^+.

For pig heart enzyme it was shown that inhibition by NADH in the reverse reaction could be prevented by NAD^+ [15], implying that the relative affinity for NAD^+ and NADH is important with respect to over-reduction. Therefore, the over-reduction may not only be related to the redox potentials of the different enzymes but the relative affinities and reaction rates of NAD^+ and NADH at the EH_2 level may contribute as well. With respect to *A. vinelandii* wild-type and mutated enzymes, it can be inferred from the diaphorase activity that the affinity for NADH is not very different among the enzymes. A similar conclusion is drawn from rapid reaction kinetics of E_{ox} reacting with NADH. Furthermore, the reoxidation reaction of EH_2 by NAD^+ shows similar K_d values for enzymes Glu455 → Gln, His450 → Ser and wild-type enzyme. These data taken together indicate that the over-reduction and thus susceptibility to inhibition by NADH, is directly related to differences in redox potentials. The relation between

the relative redox potentials of the mutated enzymes and the susceptibility to inhibition by NADH supports this hypothesis.

Assuming that the change in the redox potential with pH for the *A. vinelandii* enzyme relative to NADH is similar to that of pig heart enzyme [20], over-reduction would be favoured at lower pH. The inhibition however becomes apparent at a pH where the histidine likely deprotonates (pK_a = 8.1, inferred from the pK_m AcPyAde$^+$ plot). Our results show that binding of a nucleotide raises the redox potential of the EH_2/EH_4 couple. Apparently this rise is enhanced when the histidine is deprotonated. This indicates that the protonated histidine, at the EH_2 level, plays an important role in maintaining the redox potential of the active site carefully balanced to avoid over-reduction by NADH. The catalytic properties of the Glu455-mutated enzymes lend strong support to this hypothesis. Over the entire pH range studied strong inhibition by NADH is observed for both enzymes while it was shown previously that the pK_a of the histidine in these enzymes is considerably lowered ($pK_a \leq 6.5$) when compared to wild-type [26]. This adds a second function to His450: not only does it serve as the base for deprotonation of $Lip(SH)_2$, it modulates the relative redox potentials of the disulfide/dithiol and FAD/FADH$^-$ couples which may be reflected in the markedly diminished rates of intramolecular electron transfer in the altered enzymes.

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