Nitroreductase activity of heart lipoamide dehydrogenase

C. Stanley TSAI

Department of Chemistry and Institute of Biochemistry, Carleton University, Ottawa, Ont. K1S 5B6, Canada

A novel reaction catalysed by lipoamide dehydrogenase is described. In the presence of NADH, lipoamide dehydrogenase reduces the nitro group of 4-nitropyridine and 4-nitropyridine *N*-oxide. The elution profiles from a DEAE-cellulose column for the dehydrogenase and nitroreductase activities are identical. Chemical modifications of critical amino acid residues suggest that the two activities share a common catalytic domain. Nitro reduction catalysed by lipoamide dehydrogenase was monitored spectrophotometrically and chromatographically. The major product from the enzymic reduction of 4-nitropyridine was isolated and characterized structurally as *NN*-bis(pyridinyl)hydroxylamine, which is formed presumably via 4-hydroxyaminopyridine in a four-electron redox reaction.

INTRODUCTION

Previous studies by Dixon (1971*a,b*) showed that free reduced flavin reacted with a wide variety of acceptors. A combination of flavin with apoenzymes greatly alters its acceptor specificity. Thus flavoenzymes serve as ideal systems with which to investigate the role of protein molecules in modulating functional and substrate specificities of enzymes. Lipoamide dehydrogenase (NADH:lipoamide oxidoreductase, EC 1.8.1.4) from pig heart is a dimeric flavoenzyme. It contains one molecule of FAD, a redox-active disulphide and a catalytically competent histidine residue per subunit (Massey & Palmer, 1962*a*; Williams, 1976; Tsai *et al.*, 1982). The enzyme catalyses a number of NADH-linked reactions, utilizing acceptors of varied structures.

During my search for new electron acceptors with which to investigate the functional specificity of lipoamide dehydrogenase, I observed that the enzyme reduces 4-nitropyridine and 4-nitropyridine *N*-oxide with NADH as an electron donor. The reduction of the nitro group by lipoamide dehydrogenase is novel and mechanistically interesting, because the nitro group can accept various numbers of electrons in the redox reactions (Rinehart, 1973). The major product from the enzymic reduction of 4-nitropyridine has been isolated and structurally characterized. A plausible mechanism for lipoamide dehydrogenase-catalysed nitroreduction is discussed.

EXPERIMENTAL

Materials

Lipoamide dehydrogenase from pig heart, NADH, DL-lipoamide and iodoacetic acid were obtained from Sigma Chemical Co. The enzyme was checked for purity by electrophoresis, spectrometry (A_{280}/A_{450}) and atomic absorption (for trace Cu^{2+} ion) before use (Tsai, 1980). Pyridine *N*-oxide and phenylglyoxal monohydrate were provided by Aldrich Chemical Co. and purified respectively by vacuum distillation and recrystallization before use. Methylene Blue was supplied by Fischer Scientific Co. DEAE-cellulose (DE 52) was the product of Whatman Laboratories. Silica-gel 60F-254 t.l.c. plates were purchased from BDH Chemicals.

Dihydrolipoamide was prepared from DL-lipoamide by NaBH_4 reduction (Reed *et al.*, 1958). 4-Nitropyridine *N*-oxide was synthesized by nitration of pyridine *N*-oxide (Ochiai, 1953). Crystallization from acetone or hot water yielded pale-yellow needles, m.p. 156 °C. Deoxygenation was carried out by refluxing 4-nitropyridine *N*-oxide (5.0 g) with PCl_3 (9.5 ml) in chloroform (75 ml) at 70–80 °C for 1.0 h (Ochiai, 1953). After cooling, the reaction mixture on ice was neutralized with NaOH and extracted with chloroform. The chloroform extract was dried with anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. Crystallization of the residue was effected with light petroleum (b.p. 35–60 °C), and recrystallization with the same solvent yielded 4-nitropyridine as pale-yellow plates, m.p. 50 °C (m.s. M^+ at m/z 124; i.r., ν_{NO_2} 1345 and 1540 cm^{-1} ; ^1H n.m.r., δ 8.16 and 8.94 p.p.m.)

Enzymic assays and kinetic studies

Lipoamide dehydrogenase activity was assayed by measuring the decrease in A_{340} of the reaction mixture (1.0 ml) containing enzyme, 0.10 mM-NADH and 1.0 mM-DL-lipoamide in 0.10 M-potassium phosphate buffer, pH 7.0. Nitroreductase activity of lipoamide dehydrogenase was measured spectrophotometrically at 340 nm by the difference in rates of NADH oxidations in the presence and in the absence of nitro compounds. The reaction mixtures (1.0 ml) contained enzyme and 0.10 mM-NADH, with or without 0.10 mM nitro compound, in 0.10 M-phosphate buffer, pH 7.0. Alternatively, the reduction of 4-nitropyridine *N*-oxide (0.10 mM) with dihydrolipoamide (0.25 mM) was monitored spectrophotometrically at 320 nm.

For kinetic studies, the reaction mixture contained 196 nM lipoamide dehydrogenase and 0.01–0.10 mM-NADH, with or without 0.02–0.40 mM-4-nitropyridine or -4-nitropyridine *N*-oxide, in 0.10 M-potassium phosphate buffer, pH 7.0, at 25 °C. The decrease in A_{340} was monitored in a Beckman model 25 spectrophotometer or a Gilford model 240 spectrophotometer. Initial rates (v), after correction for the oxidase reaction (without the nitro compound), were analysed by the equation:

$$v = \frac{VAB}{AB + K_aB + K_bA + K_{ia}K_b}$$

Fig. 1. Lipoamide dehydrogenase (196 nM)-catalysed oxidation of NADH (0.10 mM) (●) in the presence of pyridine *N*-oxide or 4-nitrophenol (0.10 mM, Δ), 4-nitropyridine (0.10 mM, □) or 4-nitropyridine *N*-oxide (0.10 mM, ○) at pH 7.0

where V is the maximum velocity, K_a and K_b are Michaelis constants for NADH (A) and the nitro compound (B), and K_{ia} is the inhibition constant for NADH.

Chemical modifications of enzymes

Complexing with arsenite (Massey & Palmer, 1962b) and reductive carboxymethylation (Tsai *et al.*, 1981a) of the redox-active disulphide group were carried out as described in the references cited. Photo-oxidation was

Table 1. Rate of O_2 consumption during nitroreduction

O_2 consumption was measured manometrically with a Gilson differential respirometer. The pseudo-first-order rate constants, k , are given for reaction mixtures containing 196 nM enzyme and 0.10 mM-NADH with or without 0.10 mM nitro compound at pH 7.0.

	$10^3 k$ (min^{-1})
NADH	7.01 ± 0.58
NADH + 4-nitropyridine	6.10
NADH + 4-nitropyridine <i>N</i> -oxide	7.46

sensitized with Methylene Blue (Tsai *et al.*, 1982). Phenylglyoxalation of arginine residues and acetimidination of lysine residues were performed by the procedures of Tsai *et al.* (1981b). Amidation with propylamine was effected with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (Tsai *et al.*, 1983). At time intervals samples were withdrawn, filtered through a column of Bio-Gel P6 and assayed for enzymic activities.

Chromatographic analyses

Anion-exchange column chromatography, which separated three lipoamide dehydrogenase isoenzymes (Stein & Stein, 1965; Cohen & McManus, 1972), was carried out by loading a dialysed solution of the enzyme on a column (2.6 cm $\times$ 36 cm) of DEAE-cellulose (DE 52). Flavoprotein was eluted with a linear gradient of phosphate buffer, pH 7.5, from 0.050 M to 0.10 M in the first step and a salt gradient of 0–0.30 M-NaCl in 0.10 M-phosphate buffer, pH 7.5, in the second step.

For t.l.c. analyses of reaction products, the enzymic reduction was performed in a mixture containing

Fig. 2. Reduction of 4-nitropyridine *N*-oxide (1.0 mM) in the presence of dihydrolipoamide (2.0 mM) by lipoamide dehydrogenase (196 nM) at pH 7.0

Reduction is shown by a decrease in the A_{315} at 0, 5, 15 and 45 min. The inset shows that rates of the nitroreduction (decrease in A_{315}) are linearly correlated with enzyme concentrations.

Fig. 3. Anion-exchange (DEAE-cellulose) chromatography of lipoamide dehydrogenase

The column chromatography was performed as described in the text: ---- buffer (first step) and salt (second step) gradients. Each fraction was assayed for protein (A_{280} ; —), FAD content (A_{455} ; -----), lipoamide dehydrogenase (○) and nitroreductase (△) activities.

0.10 mg of lipoamide dehydrogenase, 10 mg of NADH and 5.0 mg of 4-nitropyridine per ml of 0.050 M-phosphate buffer, pH 7.0, at 25 °C. At time intervals, samples were withdrawn and immediately immersed in a boiling-water bath to stop the reaction. A 10 μ l portion of the heated reaction mixture was applied on a silica-gel plate, which was developed with ethanol/acetone (1:1, by v/v). The products were detected under a u.v. lamp.

Isolation of the major reduction product

For this, a scaled-up reaction was carried out. The reaction mixture, containing 2.0 mg of enzyme, 200 mg of NADH and 50 mg of 4-nitropyridine in 3.0 ml of 0.050 M-phosphate buffer, pH 7.0, was kept at room temperature for 20–30 h. The product was precipitated, and was collected by suction filtration and washed twice with cold water; yield, 8–12 mg; m.p. 180–182 °C. Elementary analysis gave: C, 63.70%; H, 5.33%; N, 22.21%; O, 8.76%.

Table 2. Effects of chemical modifications on nitroreductase activity

All modifications were carried out for 30 min. The controls without the modifying reagents were prepared in parallel runs.

Modification	Activity (% of control)	
	Dehydrogenase	Nitroreductase
Reductive carboxy-methylation	0	0
Arsenite chelation	0	16
Sensitized photo-oxidation	3	10
Phenylglyoxalation	83	98
Acetimination	129	107
Amidation	98	112

Spectroscopic and analytical methods

Visible and u.v. spectra were taken with a Cary 14 spectrophotometer. I.r. spectra were recorded with a Perkin-Elmer model 237B i.r. spectrophotometer. Mass spectra were taken with a VG 7070E mass spectrometer (Mass Spectrometer Laboratory, University of Ottawa) equipped with a DEC PDP8 data system. N.m.r. spectra were obtained with a Varian XL-200 n.m.r. spectrometer equipped with Nicolet Fourier transform and TT-100 data system. Enzyme concentrations were determined spectrometrically at 455 nm by using an absorption coefficient of $11.3 \text{ nm}^{-1} \cdot \text{cm}^{-1}$ and calculated on the basis of FAD content. pH was adjusted with a Fisher model 825 MP Accumet pH-meter, and ionic strength of eluates was measured with a Radiometer CDM2d conductivity-meter.

RESULTS

Lipoamide dehydrogenase was reduced by NADH to form a reduced enzyme. In the absence of external electron acceptors, the reduced enzyme was re-oxidized aerobically, resulting in oxidation of NADH with concomitant O_2 consumption (oxidase activity). The rate of NADH oxidation was facilitated by 4-nitropyridine and 4-nitropyridine *N*-oxide (Fig. 1), but not by pyridine *N*-oxide or 4-nitrophenol. Table 1 shows that 4-nitropyridine and its *N*-oxide facilitated NADH oxidation without stimulating O_2 consumption. In the absence of the enzyme neither 4-nitropyridine nor its *N*-oxide promoted NADH oxidation.

The reduction of 4-nitropyridine *N*-oxide by lipoamide dehydrogenase, with dihydrolipoamide as an electron donor, was demonstrated by the time-dependent decrease in the characteristic absorption of 4-nitropyridine *N*-oxide at 315 nm (Fig. 2). Furthermore, the rate of reduction was accelerated with increased enzyme concentrations (Fig. 2 inset).

Fig. 3 shows the intimate correspondence between the elution profiles for the dehydrogenase and nitroreductase

Table 3. Kinetic parameters of lipoamide dehydrogenase-catalysed nitroreductions at pH 7.0

Average values for three determinations ($\pm 15\%$) are given. $[E]_t = 196 \text{ nM}$; A (a) and B (b) refer to electron donors and nitro compounds respectively.

	A . . . B . . .	NADH 4-Nitropyridine	NADH 4-Nitropyridine <i>N</i> -oxide	Dihydrolipoamide 4-Nitropyridine <i>N</i> -oxide
V ($\mu\text{M}/\text{min}$)		21.7	37.9	8.33
K_a (μM)		30.4	14.2	41.7
K_b (μM)		43.0	164	250
K_{ia} (μM)		2.78	13.4	8.75
$V/[E]_t$ (min^{-1})		111	193	42.5

(reduction of 4-nitropyridine) activities for all isoenzymes, which were resolved by an anion-exchanger (Stein & Stein, 1965; Cohen & McManus, 1972). To substantiate further that lipoamide dehydrogenase is responsible for the nitroreduction, chemical modifications of critical amino acid residues were carried out to correlate the

dehydrogenase and the nitroreductase activities (Table 2). Monomerization of the active-site disulphide by reductive carboxymethylation (Tsai *et al.*, 1981a) and complexing thereof with arsenite (Massey & Palmer, 1962b) abolished the two activities. Photo-oxidative destruction of histidine residues sensitized by Methylene Blue (Tsai *et al.*, 1983) was detrimental to both activities. Phenylglyoxalation of arginine residues, acetimidation of lysine residues and amidation of carboxy groups did not significantly affect the enzymic activities. These parallel chemical effects suggest that the nitroreduction and the dehydrogenation by lipoamide dehydrogenase share common structures for their catalytic activities.

Table 3 summarizes kinetic parameters for lipoamide dehydrogenase-catalysed reductions of 4-nitropyridine and 4-nitropyridine *N*-oxide. The progress of the enzymic reduction of 4-nitropyridine was followed by monitoring its product formation by silica-gel t.l.c. 4-Nitropyridine moved close to the solvent front, whereas NADH remained on the origin. Three products, with R_F values of 0.34, 0.58 and 0.78, were formed (Fig. 4). The compound with R_F 0.58 was the major product that accumulated with prolonged reaction time. It was readily isolated from a scaled-up reaction mixture, although attempts to isolate the other two products were unsuccessful. The isolated product moved on a silica-gel t.l.c. plate as a single spot with R_F values of 0.58 ± 0.02 or 0.16 ± 0.04 in ethanol/acetone (1:1, v/v) or acetone/ethyl acetate (2:3, v/v) as the developing solvent respectively. The absence of ν_{NO_2} at 1345 and 1540 cm^{-1} in its i.r. spectrum (Table 4) implies the reduction of the nitro group. Spectral properties of the major product are summarized in Table 4.

Fig. 4. T.l.c. analysis of lipoamide dehydrogenase-catalysed reduction of 4-nitropyridine

The nitroreduction was carried out as described in the text. Samples were withdrawn (from left to right) at 0, 2, 5, 10 and 30 min after the enzymic reaction was stopped; $10 \mu\text{l}$ samples were applied on a silica-gel t.l.c. plate, which was developed with ethanol/acetone (1:1, v/v). With prolonged reaction time, the compound with R_F 0.58 accumulated.

Table 4. Some spectral characteristics of the major product from enzymic reduction of 4-nitropyridine

Spectrum	Parameter	Characteristics
U.v. (CH_3OH)	λ_{max} (nm)	290
I.r.	ν (cm^{-1})	1505, 1535, 1590, 1640
M.s.	m/z	187 (M^+), 170 ($M^+ - 17$), 146 ($M^+ - 41$), 95 (base), 94 ($\text{C}_5\text{H}_6\text{N}_2^+$), 78 ($\text{C}_5\text{H}_4\text{N}^+$), 67 (94-HCN) 51 (78-HCN) 39 (C_3H_3^+)
^1H n.m.r. ($\text{C}^2\text{H}_3\text{O}^2\text{H}$)	δ (p.p.m.)	8.29 (d, 4H), 8.31 (d, 4H)
^{13}C n.m.r. ($\text{C}^2\text{H}_3\text{O}^2\text{H}$)	δ (p.p.m.)	110.87 (s, 6C), 150.96 (s, 4C)

DISCUSSION

Although the nitro group is not listed as one of the substrate functional groups for flavoenzymes (Walsh, 1982), nitroalkanes are oxidized by glucose oxidase (Porter & Bright, 1977) and 2-nitropropane dioxygenase (Kido & Soda, 1984). The present study demonstrates that lipoamide dehydrogenase, in addition to its dehydrogenase, transhydrogenase, electrontransferase, diaphorase and oxidase activities, catalyses the reduction of 4-nitropyridine and 4-nitropyridine *N*-oxide in the presence of NADH as the electron donor. This is concluded from the following observations. (1) 4-Nitropyridine and 4-nitropyridine *N*-oxide facilitate the rate of lipoamide dehydrogenase-catalysed NADH oxidation without stimulating O_2 consumption. (2) In the absence of lipoamide dehydrogenase redox reaction takes place neither between NADH and 4-nitropyridine nor between NADH and 4-nitropyridine *N*-oxide. (3) The reduction of 4-nitropyridine *N*-oxide with dihydro-lipoamide as the electron donor is demonstrated by a time-dependent decrease in its characteristic absorption at 315 nm. (4) The reduction of 4-nitropyridine is evidenced by the time-dependent formation of three t.l.c.-distinguishable products. (5) The nitroreductase activity is co-eluted with the dehydrogenase activity of lipoamide dehydrogenase from a DEAE-cellulose column. Furthermore, both activities respond identically to chemical modifications.

The major product of lipoamide dehydrogenase-catalysed reduction of 4-nitropyridine has been isolated for structural characterization. The elementary analysis and the molecular ion (M^+) of 187 from its mass spectrum suggest that the product is a dipyridinyl derivative, $C_{10}H_9N_3O$. The fragmentation pattern of the low- m/z region of the mass spectrum suggests the presence of the pyridine ring (Porter & Baldas, 1971). This is substantiated by the 1H -n.m.r. spectrum, which indicates an equivalent substitution of the pyridine rings at the 4-position (White & Williams, 1971; Retcofsky & Friedel, 1967). The i.r. spectrum indicates the absence of the nitro group but the presence of the hydroxy and/or the amino group. These spectral characteristics are consistent with a structural assignment of *NN*-bis(pyridinyl)hydroxylamine.

In a model reaction, chemically reduced flavin promotes the reduction of nitrobenzene to nitroso-benzene and azoxybenzene, presumably via *N*-phenyl-

hydroxylamine (Gibian & Baumstark, 1971). It is likely that 4-nitropyridine is reduced by the reduced lipoamide dehydrogenase to 4-hydroxyaminopyridine, which, in turn, replaces the nitro group of another 4-nitropyridine molecule by a substitution reaction (Boulton & McKillop, 1984) to yield *NN*-bis(pyridinyl)hydroxylamine (Scheme 1). Disulphide flavoenzymes are potentially capable of catalysing one-, two- or four-electron transfer reactions (Bruice, 1980; Walsh, 1980). One- and two-electron redox reactions catalysed by lipoamide dehydrogenase have been documented (Williams, 1976; Tsai *et al.*, 1982, 1983). The present work demonstrates the reduction of 4-nitropyridine to *NN*-bis(pyridinyl)hydroxylamine catalysed by lipoamide dehydrogenase, presumably via a four-electron redox process.

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Scheme 1. A plausible mechanism for the formation of *NN*-bis(pyridinyl)hydroxylamine

EH_4 represents either two EH_2 dimer or four-electron-reduced enzyme.

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