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Dihydrolipoamide dehydrogenase from porcine heart catalyzes NADH-dependent scavenging of nitric oxide

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Abstract Dihydrolipoamide dehydrogenase (DLDH; EC 1.8.1.4) from porcine heart is capable of using nitric oxide (NO) as an electron acceptor, with NADH as the electron donor, forming nitrate in the reaction. NADPH was not effective as an electron donor. The reaction had a pH optimum near 6 and was not inhibited by cyanide or diphenyleneiodonium ions. The K_m for NADH was 10 μM, while that for NO was 0.5 μM. The rate of NO conversion was comparable to the rate of lipoamide conversion (200 μmol min^{-1} mg^{-1} protein at pH 6). Cytochrome c or myoglobin were poor electron acceptors by themselves but, in the presence of methylene blue, DLDH had an activity of 5–7 μmol min^{-1} mg^{-1} protein with these substrates, indicating that DLDH can act also as a methemoglobin reductase. While the K_m of DLDH for NO is relatively low, it is in the physiological range of NO levels encountered in the tissue. The enzyme may, therefore, have a significant role in modifying NO levels under specific cell conditions.

Keywords: Dihydrolipoamide dehydrogenase; Diaphorase; Nitric oxide; Cytochrome *c* reductase; NO scavenging

1. Introduction

Nitrosative stress is encountered in all living organisms. Reactive nitrogen species, such as the nitric oxide radical (nitrogen monooxide, NO) and the potent peroxynitrite ($ONOO^-$), formed via interaction of NO and superoxide, are the major agents responsible for nitrosative stress. Organisms use different methods to detoxify NO. Several bacteria, yeast and other primitive organisms contain flavohemoglobins possessing both heme and flavin prosthetic groups. The heme domain converts NO to nitrate in the presence of oxygen and the oxidized heme is reduced by NAD(P)H via the flavin portion of the enzyme [1,2]. Some bacteria [3] and most if not all higher plant and animal [4] species do not contain flavohemoglobins, but they contain hexacoordinate hemoglobins that can be involved in the modulation of NO levels in cells [4–7]. In mammalian tissues, NADPH-dependent NO dioxygenase-like activity, linked to a flavoprotein and presumably hemeprotein, has been reported [8]. However, specific proteins that catalyze this reaction have not been identified. It has been suggested that myoglobin can catalyze NO conversion to nitrate, the oxidized myoglobin being reduced by a methemoglobin reductase, such as the cytochrome b_5 reductase of the endoplasmic reticulum [9]. The importance of this reaction in vivo in non-vascular tissue has been questioned since, at the submicromolar concentrations of NO observed in cardiomyocytes, cytochrome *c* oxidase catalyzed conversion of NO to nitrite may be kinetically favourable over myoglobin-linked NO conversion to nitrate [10]. There have been reports of NO oxidase or dioxygenase-like activity in cytochrome *c* oxidase under a variety of different conditions [11–13] and in an unidentified protein not related to hemoglobins or cytochrome *c* oxidase in brain tissue [14].

In the present study, we report on the novel finding that NO scavenging activity is a property of dihydrolipoamide dehydrogenase. The reaction consumes NADH and has a K_m of 0.5 μM for NO. The abundance of DLDH and its high reactivity with NO suggest that it may be an important enzyme for decreasing abnormally high NO levels in cells.

2. Materials and methods

Porcine heart dihydrolipoamide dehydrogenase (DLDH) was obtained from Sigma (EC 1.8.1.4, catalogue number L 2002, 100–200 units mg^{-1} protein, 2–5 units mg^{-1} protein NADH diaphorase activity). Before measurements it was dialyzed to remove ammonium sulfate against 25 mM Tris–HCl buffer, pH 8.0, using centricon tubes. Cytochrome *c* reductase from porcine heart (CCR, liophylized powder from Sigma, marked as EC 1.6.99.3, catalogue number C 3381, 1–3 units mg^{-1} protein) was dissolved in 20 mM Tris–HCl buffer and further purified on DEAE Sephacel column (0.5 × 2 cm) using step gradient of KCl. The protein was eluted between 175 and 200 mM KCl.

NO conversion was measured using an NO electrode (NOMK2, World Precision Instruments, USA). The medium was 50 mM Tris–HCl buffer (pH 7.5) in a 2 ml vial. NO (~1 μM) was added either from the solution in the same buffer, which was prepared by bubbling from NO tank (Matheson company Inc., USA), or it was delivered by the NO donors, sodium nitroprusside (SNP) (Sigma) or sodium 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEANO) (Alexis Biochemicals). 1 mM SNP was added with continuous stirring, the vial was illuminated and NO reached a saturating concentration of 1–1.5 μM. 20 μM DEANO was added in the darkness and NO reached the same values as in the case with SNP. The sample (1–10 μl) was added, followed by the addition of NAD(P)H (0.1 mM). The reaction was followed until NO was depleted. There was no NO decrease with NAD(P)H in the absence of the sample.

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Abbreviations: DLDH, dihydrolipoamide dehydrogenase; CCR, cytochrome *c* reductase; DEANO, sodium 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide; SNP, sodium nitroprusside; DPI, diphenyleneiodonium

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Sodium phosphate or MES was used in place of Tris buffer when indicated, in particular for pH-dependence measurements. The buffer usually (in particular when NO was delivered by SNP) contained 1 mg ml^{-1} bovine Cu,Zn-superoxide dismutase (SOD) (Sigma) to prevent formation of peroxynitrite via interaction of NO and superoxide [8]. SOD did not interfere with the reaction when NO was delivered in a gaseous form (as shown in Table 4) or by DEANO (data not shown) and could be omitted. SOD did, however, reduce to zero the non-enzymatic rate of NO scavenging by superoxide, formed in the presence of flavin in the light, when SNP was used.

DLDH activity with different electron acceptors was measured in 50 mM sodium phosphate buffer, pH 7.5 or 6.0, containing 0.15 mM NADH. Acceptors used were 2 mM lipoamide, 2 mM lipoic acid (both in the presence of 0.15 mM NAD^+), 50 μM cytochrome *c*, 50 μM myoglobin (both in the absence or in the presence of 2.5 μM methylene blue), or 20 μM dichlorophenolindophenol (DCPIP). NADH oxidase activity was measured with 0.15 mM NADH alone. There was no activity of DLDH with all acceptors used if NADH was substituted by NADPH. The activity with lipoamide, lipoic acid or the NADH oxidase activity was detected at 340 nm ($\varepsilon = 6.22$ mM^{-1} cm^{-1}), the activity with cytochrome *c* at 550 nm ($\varepsilon = 19.2$ mM^{-1} cm^{-1}), with myoglobin at 580 nm ($\varepsilon = 14.4$ mM^{-1} cm^{-1}), with DCPIP at 600 nm ($\varepsilon = 21$ mM^{-1} cm^{-1}).

The inhibitors were applied 10 min before the addition of NADH (1 mM KCN, 20 μM antimycin A, 20 μM diphenyleneiodonium, DPI). *N*-ethylmaleimide (1 mM) was added to DLDH 1 h before the measurement of activity. Total protein was determined by Bradford [15].

To identify the presumed reaction product, NO_3^-, NO gas (Matheson Gas Products, Toronto) was added to 1 ml of 50 mM phosphate buffer to yield a concentration of 80–100 μM. This high NO concentration, two orders of magnitude higher than in all other experiments, was required to detect the reaction product. After adding the sample and NADH (0.1 mM), the reaction was allowed to proceed until NO was depleted. The sample was boiled for 2 min, cooled on ice and nitrate was determined using nitrate reductase from *Aspergillus* (Sigma) as described in [16]. The control sample contained no nitrate reductase. Nitrite formed was quantified by the Griess reagent.

K_m (NO) was determined in 50 mM Tris–HCl buffer, pH 7.5, from double reciprocal plots by measuring NO scavenging rates using different NO concentrations (commercial NO from the tank) and K_m (NADH) was determined by measuring NO scavenging rates at different NADH concentrations. For measurements of pH optimum we used 50 mM Na-phosphate buffer with different pH values, NO was supplied by adding SNP. At low pH values phosphate buffer was substituted by 30 mM MES.

SDS–PAGE electrophoresis has been performed using BioRad minigel system with acrylamide concentrations of 15%. Coomassie stained bands were in situ digested with modified trypsin as described [17]. Tryptic peptide mixture was purified on reverse-phase POROS R2 (20–30 μm bead size, PerSeptive Biosystems, Framingham, CA, USA) nanocolumn [18], eluted onto the MALDI probe with saturated matrix solution (2,5-dihydroxybenzoic acid in 50% (v/v) acetonitrile/ 5% formic acid) and MS/MS analysis of peptides mixture was performed by MALDI Qq-TOF mass spectrometer (Manitoba/Sciex prototype) [19,20]. Knexus automation software package (Proteometrics LLC, Canada) with ProFound search engine was used for peptide mass fingerprint analysis of MS spectra. Tandem MS spectra were analyzed by using the software *m/z* (Proteometrics Ltd., New York, NY, USA) and Sonar MS/MS (Proteometrics, Canada) search engine.

3. Results

Commercially available (Sigma) DLDH had a purity of >90%, with the main band at ~55 kDa (Fig. 1). The same major band was found in the Sigma preparation of CCR. Mass-spectrometric analysis showed that this major band was identical to DLDH. Its further purification on DEAE Sephacel column resulted in preparation containing only a small impurity caused by the presence of creatine kinase (Table 1, Fig. 1). This purified sample exhibited similar rates of NO, lipoamide and cytochrome *c* conversion as compared to the preparation of DLDH. From this (and also from activity measurements, see below), we conclude that both Sigma products are different preparations of DLDH.

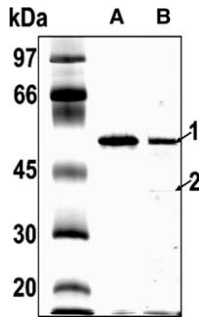

Fig. 1. Electrophoregrams of DLDH (A) and CCR (B) from porcine heart. Numbers of bands correspond to those analyzed by mass spectrometry.

Preparations of both DLDH and CCR possessed CCR and lipoamide dehydrogenase activities that were within the same range (data not shown). They also scavenged NO in the presence of NADH (Fig. 2). NADPH was not effective as a cofactor, at least within the detection range (<0.5%) of the assay. The DLDH activity with different electron acceptors is shown in Table 2. The activity with lipoamide, lipoic acid, NO and DCPIP was higher at acidic pH (6.0), while NADH oxidase, cytochrome *c* and metmyoglobin reductase activities were higher at pH 7.5. Addition of methylene blue strongly stimulated cytochrome *c* and metmyoglobin reductase activities.

The NO scavenging activity was the same either with commercial NO or with NO supplied by SNP. The activity was about twofold lower when NO was supplied by DEANO (data not shown). This may be the result of inhibition of DLDH by this compound, as has been reported for other enzymes [21]. The product of NO scavenging activity was shown to be nitrate (Table 3), indicating that DLDH operates as a diaphorase using NO effectively as electron acceptor [2]. Nitrite production was negligible in the absence of nitrate reductase.

The optimum pH for NO scavenging was 5.0–6.0 (Fig. 3), but at pH 7.5–8 the rate was still significant. The K_m for NO and NADH were 0.5 ± 0.2 and 10 ± 3 μM, respectively (Fig. 4). The activity was over 90% inhibited by 1 mM *N*-ethylmaleimide. KCN, DPI and antimycin A had no inhibitory effect (Table 4). The addition of NAD^+, to eliminate a lag phase in the reaction, was necessary for maximal activity with lipoamide and lipoic acid (data not shown) NAD^+ was inhibitory with cytochrome *c* and DCPIP (data not shown), while it had no effect with NO as substrate.

4. Discussion

Dihydrolipoamide dehydrogenase (EC 1.8.1.4) was discovered by Straub [22]. It is known as a flavoprotein with broad specificity for electron acceptors [23–25]. In addition to the activity with lipoamide, it can act as a diaphorase, transferring electrons from NAD(P)H to oxygen. It is also capable of using artificial electron acceptors such as methylene blue, DCPIP,

Table 1
Proteins identified by matrix-assisted laser desorption/ionization–time of flight tandem mass spectrometry

Identity	Band #	PMF Cover %	DB #	Taxonomy	ST
Dihydrolipoamide dehydrogenase	1	67	AAA35764 GI:181575	*Homo sapiens*	ADQPIDADVTVIGSGPGGYVAAIK LGADVTAVEFLGHVGGVGIDMEISK RPFTKNLGLEELGIELDPR VCHAHPTLSEAFR
Basic-type mitochondrial creatine kinase	2	77	NP_001816 GI:4502855	*Homo sapiens*	LFPPSADYPDLR LFPPSADYPDLRK REVENVAITALEGLK EVENVAITALEGLK TFLIWINEEDHTR GTGGVDTAAVADVYDISNIDR RGTGGVDTAAVADVYDISNIDR

Sequence tags (ST) identified by MALDI-Qq-TOF MS/MS analysis and Sonar MS/MS Ions Search engine at <10 ppm of the mass assignment error. MALDI-Qq-TOF peptide mass fingerprint (PMF) analysis was done using ProFound Search engine at <35 ppm of the mass assignment error. The accession numbers represent entries in NCBI (DB #). The band numbers correspond to the SDS–PAGE images in Fig. 1.

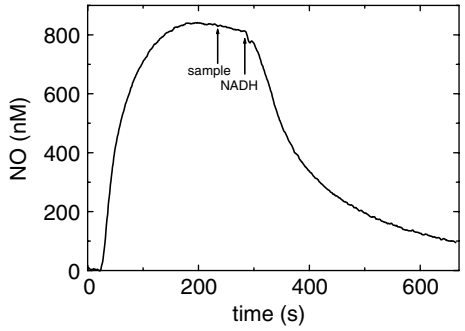

Fig. 2. Profile of the changes in NO concentration over time in the presence of DLDH and 0.1 mM NADH. Gaseous NO was bubbled into 50 mM Tris–HCl buffer (pH 7.5). A typical curve is shown. When NO was delivered by the donors (SNP or DEANO), the profiles were similar, except that NO reached an equilibrium concentration in 5–10 min and the rate of disappearance of NO was lower with DEANO.

Table 2
DLDH activity (μmol min^{-1} mg^{-1} protein) at two pH values with different electron acceptors

Acceptor	pH 6.0	pH 7.5
NO	210	87
Cytochrome *c*	0.3	0.8
Cytochrome *c* and methylene blue	5.2	6.4
Metmyoglobin	0.2	0.5
Metmyoglobin and methylene blue	3.6	4.5
Lipoamide	154	83
Lipoic acid	1.7	0.4
DCPIP	7.2	3.8
O_2	0.6	0.9

Table 3
Nitrate formation from NO by DLDH ($n = 4$)

	NO decline (nmol)	NO_2^- detected (nmol)	
		+Nitrate reductase	−Nitrate reductase
−NADH	7 ± 3	10 ± 4	7 ± 2
+ NADH	64 ± 11	52 ± 14	9 ± 4
Difference	57 ± 14	42 ± 18	2 ± 6

Gaseous NO was added in amount 80–100 nmol to 1 ml vial containing 50 mM Tris–HCl buffer (pH 7.5). After boiling the sample, it was incubated with and without nitrate reductase in the presence of its cofactors [16] and the nitrite formed was quantified.

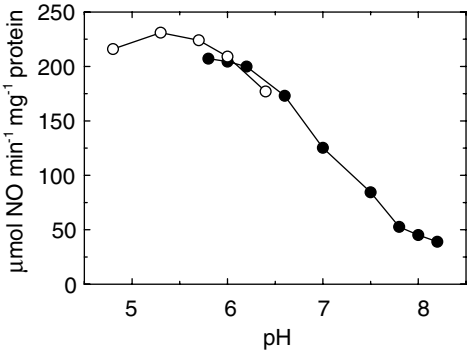

Fig. 3. pH dependence of NO conversion by DLDH. Closed symbols, 50 mM Na-phosphate buffer; open symbols, 30 mM MES buffer. Gaseous NO (1 μM) and 0.1 mM NADH were used.

ferricyanide or quinones [24,26,27]. It is also known, for at least some DLDHs, that they can use cytochrome *c* as an electron acceptor [28]. DLDH also catalyzes transhydrogenase reactions with pyridine nucleotides [26]. Two states of the enzyme have been detected: the two-electron-reduced state (EH_2), where the disulfide is reduced while the FAD is oxidized; and, the four-electron-reduced state (EH_4) where both disulfide and FAD are reduced [27]. Lipoyl substrates are reduced at the EH_2 level, while the reduction of diaphorase substrates occurs with EH_4. No physiological substrate for the EH_4 state of the enzyme has yet been identified [27], although it is possible that the discovered activity with NO is its main physiological function. No inhibition of DLDH by DPI was shown [29] and this was also revealed in our investigation with NO as the electron acceptor. This is consistent with a previous study of the inhibitory mechanisms of flavoenzymes by iodonium compounds [30], in which it was demonstrated that all known DPI-inhibited flavoenzymes function as one-electron donors, whereas DPI-insensitive flavoenzymes transfer two or more electrons during catalysis (as does DLDH).

CCR, which is not inhibited by antimycin A, was first described by Mahler [31]. The reported K_m value for NADH is 19 μM, while that of cytochrome *c* is 120 μM. NADPH is re-

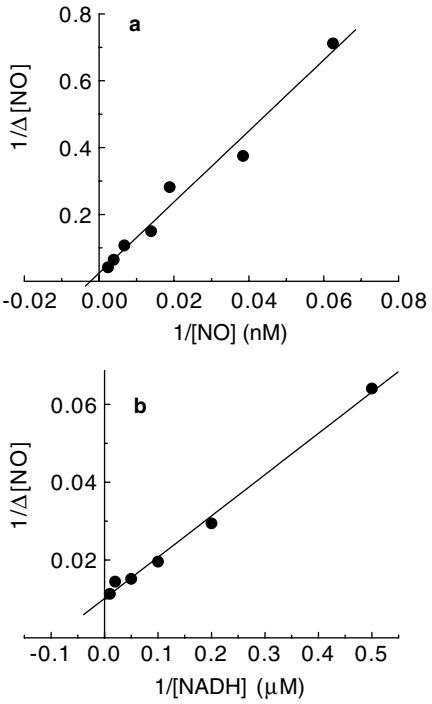

Fig. 4. Determination of K_m for NO conversion with NO (a) and NADH (b). Gaseous NO was bubbled into 50 mM Tris–HCl buffer (pH 7.5) to yield the indicated concentrations and the initial rate of its scavenging was monitored in the presence of 0.1 mM NADH (a). Using an NO concentration of 1 μM, the initial rate of NO scavenging at different concentrations of NADH was determined (b).

Table 4
Effects of addition of different compounds on DLDH activity (μmol min^{-1} mg^{-1} protein) with 1 μM NO (supplied in gaseous form) and 0.1 mM NADH in 50 mM Tris–HCl buffer, pH 7.5

Compound	Activity
No addition	85
KCN (1 mM)	96
NAD^+ (0.2 mM)	83
Superoxide dismutase (1 mg ml^{-1})	88
N-ethylmaleimide (1 mM)	11
DPI (20 μM)	87
Antimycin A (50 μM)	85

ported to be ineffective as an electron donor, while various dyes can be used as electron acceptors suggesting a diaphorase activity. The enzyme exhibits an alkaline pH optimum and is inhibited by SH-reagents, while antimycin A and cyanide have no effect. Our data demonstrate that the activity in the commercial preparation labeled as CCR is, in fact, due primarily to DLDH. Recently it has been shown that Fe^{3+} ions, complexed to physiological ligands, are readily reduced by DLDH [32]. Fe^{3+} complexes can be reduced also by glutathione reductase, CCR and cytochrome P450 reductase but with a lower efficiency [33].

In the reaction with NO, nitrate was detected as the end product, suggesting that DLDH catalyzes the conversion of NO to nitrate, indicating that the reaction proceeded in a similar fashion to what has been observed with hemoglobin-linked reactions [2,9] as opposed to a cytochrome *c* oxidase catalyzed reaction, which produces nitrite [11,12]. The formation of nitrate indicates that the reaction is oxygen-dependent. DLDH affinity to NO is relatively high (K_m 0.5 μM), but the K_m is still higher than the nanomolar NO levels normally experienced under physiological conditions in animal tissues and bacteria [13,34]. The K_m values for NO in flavohemoglobins of bacteria and yeasts are slightly lower, being in the range of 0.1 to 0.25 μM [34]. The DLDH activity with NO at physiological pH (7–7.5) is about one order of magnitude higher (80–100 μmol min^{-1} mg^{-1} protein) than that observed for *Escherichia coli* flavohemoglobin acting as an NO dioxygenase (8–11 μmol min^{-1} mg^{-1} protein) [35]. Taking into account the wide distribution of DLDH in cells, it may be possible that this enzyme can be effective in scavenging NO, where the localized NO concentrations may be excessively high. The methemoglobin (and cytochrome *c*) reductase activity of DLDH indicates that it may also reduce hemeproteins from the ferric to ferrous state. Metleghemoglobin reductase in legume plant nodules has very high sequence homology to DLDH [36]. The rate of cytochrome *c* and myoglobin reduction is higher in the presence of methylene blue, which may indicate that in vivo it proceeds in the presence of some low-molecular weight electron acceptor.

Considering the localization of DLDH in tissue, this NO scavenging activity would be present in both the cytosol and mitochondria. The greatest proportion of DLDH activity (90%) is in mitochondria, where it serves as a component of pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and glycine decarboxylase complexes [37]. In mitochondria, cytochrome *c* oxidase is reversibly inhibited by NO, converting it to nitrite [13]. Mitochondria contain a nitric oxide synthase [38] that results in NO production in mitochondria [39], with possible functional implications for mitochondrial metabolism [40]. The physiological levels of NO in porcine heart mitochondria are considered to be low [41], although there is still some controversy in their estimation due to the contribution of cytosolic NO synthase [42]. The abundance of DLDH in mitochondria and its high reactivity suggest that it can readily compete with the cytochrome *c* oxidase interaction with NO.

In conclusion, our data indicate that DLDH is capable of converting nitric oxide to nitrate utilizing NADH in the reaction. The rate of this reaction is comparable to the rate at which lipoamide reacts with the enzyme, and it exceeds the rate of other DLDH diaphorase reactions indicating that the NO scavenging activity of DLDH may be a physiologically relevant reaction of this enzyme.

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