METHYLMALONATE SEMIALDEHYDE DEHYDROGENASE FROM BACILLUS SUBTILIS: SUBSTRATE SPECIFICITY AND COENZYME A BINDING
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Methylmalonate semialdehyde dehydrogenase (MSDH) belongs to the CoA-dependent aldehyde dehydrogenase (ALDH) subfamily. It catalyzes the NAD-dependent oxidation of methylmalonate semialdehyde (MMSA) to propionyl-CoA via the acylation and deacylation steps. MSDH is the only member of the ALDH superfamily that catalyzes a \( \beta \)-decarboxylation process in the deacylation step. Recently, we demonstrated that the \( \beta \)-decarboxylation is rate-limiting and occurs before CoA attack on the thiopropionylenzyme intermediate. Thus, this prevented determination of the transthioesterification kinetic parameters. Here, we have addressed two key aspects of the mechanism: 1) the molecular basis for recognition of the carboxylate of MMSA; and 2) how CoA binding modulates its reactivity. We substituted two invariant arginines, Arg124 and Arg301, by Leu. The second-order rate constant for the acylation step for both mutants was decreased by at least 50-fold, indicating that both arginines are essential for efficient MMSA binding through interactions with the carboxylate group. To gain insight into the transthioesterification, we substituted MMSA with propionaldehyde (PPA), as both substrates lead to the same thiopropionylenzyme intermediate. This allowed us to show that: 1) the \( pK_{\text{app}} \) of CoA decreases by approx. 3 units upon binding to MSDH in the deacylation step; and 2) the catalytic efficiency of the transthioesterification is increased by at least \( 10^4 \)-fold relative to a chemical model. Moreover, we observed binding of CoA to the acylation complex, supporting a CoA binding site distinct from that of NAD(H).

INTRODUCTION

Methylmalonate semialdehyde dehydrogenases (MSDHs) belong to the CoA-dependent aldehyde dehydrogenases (ALDHs) which, together with their hydrolytic counterparts, make up the ALDH superfamily. ALDHs are known to be involved in many essential biological functions such as intermediary metabolism, detoxification, osmotic protection and cellular differentiation. The enzymes catalyse the NAD(P)-dependent oxidation of a wide variety of aldehydes to their corresponding non-activated or CoA-activated acids via a common two-step chemical mechanism. The acylation step leads to formation of a thioacylenzyme intermediate which then undergoes nucleophilic attack by a water or CoA molecule. Despite mechanistic similarities, previous studies have highlighted major differences in the kinetic mechanism of ALDHs depending on the nature of the deacylation step. In hydrolytic ALDHs, kinetic data support an ordered sequential mechanism in which NAD(P)H dissociates last (1-4). By contrast, CoA-dependent ALDHs exhibit a ping-pong mechanism in which the release of the reduced cofactor occurs before the transthioesterification step (5,6). Whereas mechanistic and structural aspects have been studied extensively for hydrolytic ALDHs (see, e.g. (7-14)), considerably less is known about CoA-dependent ALDHs.
MSDHs are present in a wide variety of organisms ranging from bacteria and archaea to plants and mammals where they are described to play a diversity of roles. The MSDHs from *Pseudomonas aeruginosa* (15,16) and *Streptomyces coelicolor* (17) are involved in valine catabolism, while in *Bacillus subtilis* (18) and *Rhizobium leguminosarium* bv. *viciae* (19), MSDH is putatively implicated in myo-inositol catabolism. In mammals, MSDH is a mitochondrial enzyme that participates in the distal portions of the valine and pyrimidine catabolic pathways (20). Over the last two decades, a possible correlation of organic acidemia with MSDH deficiency has been explored (21,22). For example, Roe and co-workers (23) proposed that a psychomotor delay associated with methylmalonyl aciduria is a direct consequence of a lack of MSDH activity. More recently, it was suggested that MSDH may play a role in root development and leaf sheath elongation in rice (24).

Recently, our group undertook a study of the catalytic mechanism of MSDH from *Bacillus subtilis* (25). The homotetrameric enzyme catalyzes the NAD+-dependent oxidation of methylmalonate semialdehyde (MMSA) and malonate semialdehyde to propionyl- and acetyl-CoA, respectively. Therefore, MSDH is the only member of the ALDH superfamily known to date to catalyse a β-decarboxylation in addition to the transthioesterification step. From the detailed mechanistic characterization of the MSDH-catalyzed reaction, our studies suggested that NAD binding elicits a structural imprinting of the apoenzyme which may explain the lag-phase observed in the activity assay. Half-site reactivity for MSDH was also demonstrated, with two subunits being active per tetramer. Finally, kinetic studies showed that: i) the rate constant associated with the acylation step is high ($k_{ac} > 1000 \text{ s}^{-1}$); ii) the rate-limiting β-decarboxylation step occurs on the thioacylenzyme intermediate after NADH release and before transthioesterification; and iii) bicarbonate is the end-product of the thioacylenzyme decarboxylation (25). Together, these results have led to a proposal for the catalytic mechanism (Scheme 1).

Nonetheless, several questions about key aspects of the MSDH-catalyzed reaction remain. This is the case for the molecular factors which are responsible for the recognition of a substrate with a carboxylate group. It is not also known whether binding of CoA to MSDH activates its thiol group. Indeed, the rate-limiting step of MSDH occurs before the transthioesterification with MMSA and thus prevented measurement of the pK$_{app}$ of CoA.

In the present paper, two invariant Arg residues, expected to stabilize the binding of MMSA via interactions with its carboxylate group, were substituted by Leu. The kinetic data for R124L, R301L and R124L/R301L MSDHs, are described. The results obtained under pre-steady conditions show that both Arg residues participate not only in MMSA binding via stabilizing interactions between the guanidinium groups and the carboxylate, but also in formation of an efficient MSDH/NAD/MMSA ternary complex. When propionaldehyde (PPA), a decarboxylated analogue of MMSA which leads to the same thiopropionyl enzyme intermediate, was used as alternate substrate, the rate-limiting step of the reaction catalyzed by R124L MSDH is now associated with the transthioesterification process. This gave the opportunity to determine the pK$_{app}$ of CoA in the deacylation step which shifts from approx. 9.6 to 6.8. When compared to a chemical model, the catalytic efficiency of the transthioesterification for the wild-type MSDH is at least 10$^4$-fold increased. Therefore, the CoA binding to MSDH optimizes its positioning relative to the thioacylenzyme intermediate and favors a significant decrease of the pK$_{app}$ of its thiol group. Moreover, we observed that for the wild-type MSDH (and in a lesser extent for R124L and R301L MSDHs) the catalytic efficiency of the acylation step increased in the presence of CoA, due to an enhancement in the apparent affinity of the enzyme for PPA. These data show for the first time, that the CoA-binding site is distinct from that of NAD.

**EXPERIMENTAL PROCEDURES**

**Materials**

NAD was purchased from Roche (Mannheim, Germany). CoA, propionaldehyde, pyruvate, lactate dehydrogenase (LDH) were sourced from Sigma (St. Louis, MO, U.S.A.). Desulfo-CoA was prepared as described by Chase et al., (26). MMSA was synthesized as described by Kupiecki and Coon (27). Its concentration was determined by titration with MSDH.
Site-directed mutagenesis, production and purification of wild-type and mutated MSDHs from *B. subtilis*

Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Wild-type, R124L, R301L and R124L/R301L MSDHs were produced and purified using a procedure described previously (25). Enzyme concentrations were determined spectrophotometrically by using molar absorption coefficients of $2.04 \times 10^5$ M$^{-1}$cm$^{-1}$ at 280 nm for wild-type and mutated MSDHs. In the text, enzyme concentrations are expressed per monomer (normality, N).

**Kinetic parameters for wild-type and mutated MSDHs under steady-state conditions**

Initial rate measurements were carried out at 30 °C on a SAFAS UV mc2 spectrophotometer, by following the appearance of NADH at 340 nm in 50 mM potassium phosphate (pH 8.2). Prior to kinetic measurements in the presence of MMSA, R301L MSDH was pre-incubated at 30 °C with 2 mM NAD, whereas R124L and R124L/R301L MSDHs were pre-incubated with 2 mM NAD and 0.5 mM CoA, respectively. When PPA was used as substrate, wild-type and all mutated MSDHs were pre-incubated at 30 °C with 2 mM NAD. In all the pre-incubated mixtures, the concentration of MSDHs was 160 µN. The initial rate data were fit to the Michaelis–Menten equation using non linear least-squares regression analysis to determine the $k_{cat}$ and $K_M$ values. All $K_M$ values were determined at saturating concentrations of the other substrates: 1 mM MMSA, 1 mM NAD and 3 mM CoA for R124L and R301L MSDHs; 0.05 M PPA, 1 mM NAD and 0.5 mM or 2 mM CoA for wild-type and R124L MSDHs. In the case of R301L MSDH, the substantial increase in the $K_M$ for CoA prevented accurate determination of $K_M$ values for NAD and PPA. For the pH-dependence studies, data were collected at 30 °C over a pH range of 5.00–8.75 with a reaction buffer consisting of 22 mM succinic acid, 29 mM imidazole and 29 mM diethanolamine adjusted with NaCl to a constant ionic strength of 0.06 M (buffer A), and analyzed as described by Marchal, et al. (4).

**Pre-steady-state kinetic measurements**

Pre-steady-state kinetic analyses were carried out on a SX18MV-R stopped-flow apparatus (Applied PhotoPhysics) and collected data were analyzed using the SX18MV-R software package.

**Kinetics of the acylation step and of NADH dissociation of R124L, R301L and R124L/R301L MSDHs with MMSA as substrate**

To study the acylation step, progress curves of NADH production were recorded at 340 nm and at 30 °C in 50 mM potassium phosphate (pH 8.2), in the absence of CoA or at subsaturating concentration of CoA (i.e. 50 µM) for R124L and R124L/R301L MSDHs. One syringe was filled with 16 µM MSDH and 1 mM NAD (after mixing) and the other contained MMSA at various concentrations. No saturation was observed irrespective of the mutated MSDH, and therefore $k_2$ values were estimated from the slope of the linear part of the $k_{obs} = f ([\text{MMSA}])$ profile.

To evaluate the rate of NADH dissociation from the thioacylenzyme–NADH complex, the coupled pyruvate/LDH assay was used as an NADH trapping system. One syringe was filled with 16 µM MSDH and 1 mM NAD, and the other contained 0.5 mM MMSA (which is a subsaturating concentration), 16 µM LDH and 10 mM pyruvate. Experiments were carried out in the absence of CoA (or at a subsaturating concentration of CoA, i.e. 50 µM for R124L and R124L/R301L MSDHs) in 50 mM potassium phosphate (pH 8.2) at 30 °C. Data were fit to a biphasic expression.

**Kinetic properties of wild-type, R124L, R301L and R124L/R301L MSDHs with PPA as substrate**

Kinetic analyses of the acylation step were carried out at 30 °C in 50 mM potassium phosphate (pH 8.2). For this, progress curves of NADH production were recorded at 340 nm, in the absence of CoA or in the presence of desulfo-CoA. The $k_2$ values were obtained by non linear least-square regression of $k_{obs}$, measured at sub-saturating concentrations of substrate PPA, except for the wild-type MSDH for which the second-order rate constant $k_2$ was deduced from the $K_{app}$ and the $k_{obs\ max}$ values. Alternatively, when CoA was added to the reaction mixture, the burst of NADH production associated with acylation was selectively monitored using fluorescence resonance energy transfer (FRET). Following selective excitation of MSDH Trp residues at 295 nm, the fluorescence emission of NADH was measured.
using a 395 nm cut-off filter. In this experiment, one syringe contained 16 µN wild-type or mutated MSDHs and 1 mM NAD, and the other contained PPA at various concentrations. In the experiments where CoA and desulfo-CoA were used, they were added to the syringe containing the enzyme. Data were fit to equation (1) to determine $k_{ac}$ and $K_{app}$ values, where $S$ represents the substrate PPA.

$$k_{ac} = \frac{(k_{ac\ max}[S])}{(K_{app} + [S])} \tag{1}$$

For wild-type and R124L MSDHs, the pH-dependence of the acylation rate constant was studied at 30 °C over a pH range 5.00–8.75 in buffer A. One syringe was filled with 16 µN MSDH, 1 mM NAD and 500 µM CoA or 250 µM desulfo-CoA and the other contained 0.5 M PPA. Formation of NADH was measured by monitoring the FRET signal using a 395 nm cut-off filter. Data were analysed as described by Marchal, et al. (4).

The rate of NADH dissociation from the thioacylenzyme–NADH complex was determined as described above with MMSA as substrate. One syringe was filled with 16 µN MSDH, 16 µN LDH and 10 mM pyruvate, and the other contained 1 mM NAD and 0.5 M PPA. Experiments were carried out in the absence of CoA for R124L and R301L MSDHs in 50 mM potassium phosphate (pH 8.2) at 30 °C. For the wild-type MSDH, desulfo-CoA was added to the syringe containing PPA and NAD. Data were fit to a biphasic expression.

**RESULTS**

**Rationale for the mutations**

On the basis of sequence comparison of MSDHs to those of other ALDHs (including hydrolytic and CoA-dependent ALDHs) two invariant Arg residues in MSDHs, i.e. Arg124 and Arg301, appear to be only conserved in non-phosphorylating glyceraldehyde-3-phosphate dehydrogenases (GAPNs) (Figure 1). In GAPN from *Streptococcus mutans*, these Arg residues were shown to be critical in the binding of D-glyceraldehyde 3-phosphate through stabilizing interactions with the C-3 phosphate (13,28). Moreover, the X-ray structure of the MSDH from *B. subtilis* in complex with NAD (RCSB Protein Data Bank accession number 1t90) superimposes well on the GAPN structures (accession numbers 1qi6 and 2qe0). In particular, Arg124 and Arg301 are found on the same secondary structural elements. Therefore, it was reasonable to postulate for MSDH a major role for these residues in the binding of a substrate such as MMSA, which bears a carboxylate group. In order to validate this assumption, the roles of Arg124 and Arg301 have been probed by site-directed mutagenesis. Introduction of a residue such as leucine at positions 124 and 301 would provide experimental support for the role of the electrostatic interactions between the guanidinium groups of Arg124 and Arg301 and the carboxylate group of MMSA, while keeping the hydrophobic properties of the aliphatic part of an arginine side-chain.

**Kinetic properties of R124L, R301L and R124L/R301L MSDHs with MMSA as substrate**

The lag-phase exhibited by wild-type MSDH during activity assays with MMSA as substrate was eliminated by preincubating the apoenzyme with NAD, as described previously (25). A similar behaviour was observed for R301L MSDH but not for R124L and R124L/R301L MSDHs, for which incubation with both NAD and CoA was required. Therefore, prior to any kinetic studies, R301L MSDH was preincubated with 2 mM NAD, and R124L and R124L/R301L MSDHs with 2 mM NAD and 500 µM CoA. For the wild-type MSDH-catalyzed reaction, the rate-limiting step was previously shown to be associated with the β-decarboxylation process within the deacylation step (25). Therefore, before interpreting the kinetic data of the mutated MSDHs, it was necessary to determine whether the rate-limiting step was the same as the wild-type MSDH. Accordingly, fast kinetic experiments were carried out with the three mutated MSDHs at pH 8.2 and 30 °C in the presence of 2 mM NAD and subsaturating concentrations of MMSA (i.e. 2 mM) and, in the case of R124L and R124L/R301L MSDHs, CoA (50 µM after dilution). A burst magnitude of 2 moles of NADH per mole of tetramer was observed, irrespective of the mutated MSDH. Therefore, these data clearly indicate that all mutated MSDHs also exhibited half-site reactivity, as described for the wild-type MSDH. More importantly, the $k_{obs}$ values determined at 2 mM MMSA were 82-, 36- and 150-fold higher than the $k_{cat}$ values for R124L, R301L and R124L/R301L MSDHs, respectively. These data indicate that the rate-limiting step still takes place after hydride transfer.
MMSA is at least 37-fold lower than the MSDH, the wild-type MSDH (Table 2). For R124L/R301L, the estimated $k_2$ value is 512-fold smaller than the $k_2$ value for the wild-type MSDH (Figure 2). For R124L and R301L MSDHs as the $K_M$ cannot be equated with the dissociation constant of the enzyme-substrate complex(es). By contrast, comparison of the apparent affinity constants ($K_{app}$) values for MMSA determined under pre-steady-state conditions for the wild-type and the mutated MSDHs, provides a more accurate picture of the role(s) of each arginine side-chain in MMSA binding (see below). Indeed, the rate constants associated with all the steps following hydride transfer do not contribute to the expression for $K_{app}$. Thus in this case, the $K_{app}$ values are expected to be closer to dissociation constants. For the R124L/R301L MSDH, due to lack of saturation with substrates and very low steady-state initial rates ($< 10^{-2}$ s$^{-1}$), it was not possible to determine the $k_{cat}$ and $K_M$ values.

Under pre-steady-state conditions, saturation kinetics was not observed with respect to MMSA in the acylation step up to 12 mM, irrespective of the mutated MSDH (Figure 2). For R124L and R301L MSDHs, the estimated $k_2$ values of 6000 and 5400 M$^{-1}$s$^{-1}$ are 55- and 62-fold smaller respectively than the $k_2$ value for the wild-type MSDH (Table 2). For R124L/R301L MSDH, the $k_2$ value is 512-fold smaller than that of the wild-type MSDH. The $k_{obs}$ value at 12 mM MMSA is at least 37-fold lower than the $k_{obs}$ max of the wild-type MSDH irrespective of the mutated MSDH. Therefore, the 55–512-fold decrease in $k_2$ likely reflects both a decrease of the $k_{obs}$ max value and an increase in the $K_{app}$ value.

The lower $k_{obs}$ max of the acylation step for all mutated MSDHs which was measured at pH 8.2 could have been the consequence, in part, of a significant increase in the $pK_{app}$ of Cys302. To evaluate this hypothesis, the acylation rate constant was determined for R124L MSDH, over a pH range of 5–8, at 4 mM MMSA, a subsaturating concentration for this mutant. The resulting pH-$k_2$ curve exhibited an increasing monosigmoidal profile with a $pK_{app}$ value of 6.0, similar to that observed for the wild-type MSDH, which can be assigned to Cys302, and a $k_2$ max value of $5 \times 10^3$ M$^{-1}$s$^{-1}$ at pH 8.0 (curve not shown). Therefore, substituting Arg124 with Leu does not result in an increase in the $pK_{app}$ of Cys302 compared to the wild-type MSDH. Since R124L and R301L MSDHs exhibit similar catalytic parameters, it seems reasonable to postulate a similar $pK_{app}$ of Cys302 for R301L MSDH.

NADH release from the thioacylenzyme–NADH complex is not rate-limiting for R124L, R301L and R124L/R301L MSDHs

The rate constant of NADH dissociation from the thioacylenzyme–NADH complex was determined using LDH as an NADH-trapping system and MMSA as substrate. The experiments were performed using R124L, R301L and R124L/R301L MSDHs at pH 8.2 and 30 °C under pre-steady-state conditions in the absence of CoA (R301L) or at subsaturating concentrations of CoA (50 µM after dilution) for R124L and R124L/R301L MSDHs. The resulting progress curves were fit to a biphasic expression. Under the experimental conditions used, the rate of NADH oxidation by LDH (130 s$^{-1}$) was higher than the apparent rate constants of 2.4, 1.5 and 1 s$^{-1}$ which can be assigned to release of NADH from the thioacylenzyme–NADH complex for the R124L, R301L and R124L/R301L MSDHs, respectively (Supplemental data S1). These rates – which are likely to underestimate the intrinsic rates of NADH release – are 40-, 11- and 100-fold higher than the $k_{cat}$ values. Therefore, the rate-limiting step must occur after NADH release from the thioacylenzyme–NADH complex for the R124L, R301L and R124L/R301L MSDHs.

Kinetic properties of R124L, R301L and R124L/R301L and wild-type MSDHs with PPA as substrate
Acylation with PPA leads to formation of the same thiopropionylenzyme intermediate that arises following β-decarboxylation of the MMSA substrate (see Scheme 1), and on which CoA attacks. Thus, we hoped that detailed kinetic studies of a reaction lacking the β-decarboxylation step would furnish additional insights into the transthioesterification step, provided that this step became rate-limiting. Accordingly, it was necessary to determine all the kinetic parameters for the wild-type, R124L and R301L MSDHs.

In contrast to what was observed for R124L and R124L/R301L MSDHs with MMSA as substrate, it was possible to eliminate the lag phase in the activity assay with PPA as substrate by preincubation with 2 mM NAD in the absence of CoA. Thus, in the kinetic experiments detailed below, all MSDHs including R124L and R124L/R301L mutants were preincubated with 2 mM NAD only.

**Wild-type MSDH**

The kinetic parameters determined at pH 8.2 and at 30 °C under steady-state conditions with PPA are summarized in Table 3. The wild-type enzyme displayed $K_M$ values of 33 μM and 85 μM for NAD and CoA, respectively and a $k_{cat}$ value of 12.6 s⁻¹. Under pre-steady-state conditions, fast kinetic experiments were carried out at pH 8.2 and 30 °C in the presence of 1 mM NAD and 0.05–1.40 M PPA. At 0.05 M PPA, the $k_{obs}$ value is already 8-fold higher than the $k_{cat}$ value (i.e. 103 s⁻¹ vs 12.6 s⁻¹). A substrate-saturating effect was observed, allowing determination of $k_{obs\ max}$ and $K_{app}$ values of 340 s⁻¹ and 0.15 M, respectively (Figure 3 and Table 4). Thus, addition of saturating concentration of CoA led to a 2.8-fold increase in $k_{obs\ max}$ and a 17-fold decrease in $K_{app}$ for PPA. Similar experiments were performed in the presence of 1 mM NAD and 0.5 M PPA, with CoA concentrations ranging from 0–500 μM, resulting in determination of a $K_{app}$ of 35 μM for CoA and a $k_{obs\ max}$ of 330 s⁻¹ (Supplemental data S2). The CoA-dependence of the acylation step was further supported by single turn-over experiments carried out in the presence of 1 mM NAD, 0.05 M PPA and 50 or 250 μM desulfo-CoA which is a non-active CoA analog. Under these experimental conditions, accumulation of the thiaacylenzyme intermediate (i.e. 10³ s⁻¹) could be associated with the hydrolysis of the thiaacylenzyme intermediate (i.e. 10³ s⁻¹). The $k_{obs}$ values of 45 s⁻¹ and 96 s⁻¹ are ~9 and 19-fold higher than the $k_{obs}$ determined in the absence of CoA, and 3.5- and 7.6 higher than the $k_{cat}$ value. Clearly, a desulfo-CoA-dependence of the acylation step is also observed. Moreover, all the results showed that the rate-limiting step takes place after hydride transfer and could be associated with...
NADH dissociation, transthiosterification, product release or any potential isomerisation steps.

As described earlier, the rate constant for NADH dissociation from the thioacylenzyme-NADH complex was determined using LDH as a NADH-trapping system. The experiment was performed at pH 8.2 and 30 °C under pre-steady-state conditions in the presence of 1 mM NAD, 0.5 M PPA and 50 µM or 100 µM desulfo-CoA. This yielded an apparent rate constant of 13 s⁻¹, which is similar to the kcat value, irrespective of the desulfo-CoA concentration used (Supplemental data S3). This result, together with the kinetic data obtained under steady-state conditions, showed that i) NADH dissociation occurs prior to transthiosterification, as already demonstrated for the MSDH-catalyzed reaction in the presence of MMSA, ii) NADH dissociation is likely rate-limiting for the overall reaction; and iii) the catalytic efficiency (kcat/KM) of the thioester exchange with CoA is high i.e. at least 1.5x10⁷ M⁻¹s⁻¹.

The pH-dependence of the acylation rate constant was determined in the presence of 1 mM NAD and saturating concentration of 500 µM CoA. To avoid any kcat/KM contribution in the kac-pH profile, experiments were carried out at 0.5 M PPA which was shown to be mostly saturating (3-fold the Kapp value) over the full pH range (5.00–8.75). Formation of NADH was measured by monitoring the FRET signal using a 395 nm cut-off filter. The pH-kac curve exhibited an increasing bisigmoidal profile that can be related to the contribution of two ionizable groups, one of pH Kapp 6.5 that must be deprotonated for acylation and one of pKapp 7.9 whose deprotonation increased the acylation rate by 1.9-fold with rate constants kac,i of 176 s⁻¹ and kac max of 330 s⁻¹, respectively (Figure 4). When desulfo-CoA was used instead of CoA, the pH-kac curve exhibited an increasing monosigmoidal profile that can be related to the contribution of one ionizable group with a pKapp of 6.5 and a rate constant kac of 160 s⁻¹ (Figure 4, see inset).

Additionally, the pH-dependence of the steady-state rate constant was determined at 30 °C in the presence of 1 mM NAD, 50 mM PPA (5-fold the KM value) and 500 µM CoA. Analysis of the pH-kac curve provided similar pKapp values to those extracted from the pH-kcat curve (i.e. 6.5 and 7.8), as well as a kcat of 6 s⁻¹ and kcat max of 18 s⁻¹ (Figure 5). Differences in buffer composition and ionic strength (0.15 M vs 0.06 M) likely explain the slightly higher value of kcat max (18 s⁻¹) relative to kcat (12.6 s⁻¹).

R124L and R301L MSDHs

The kinetic parameters determined at pH 8.2 and at 30 °C under steady-state conditions are summarized in Table 3. For R124L MSDH, the KM values for NAD and PPA are similar to those of the wild-type enzyme, whereas the KM for CoA is 3.9-fold higher and the kcat is 4.5-fold smaller. Substituting Leu for Arg301 led to a significant increase in KM for CoA (at least 12-fold) that prevented accurate determination of KM values for NAD and PPA. However, the likely underestimated kcat value of 3 s⁻¹ is in the same range as that determined for R124L MSDH, and smaller by only 4-fold relative to the wild-type MSDH.

Under pre-steady-state conditions, fast kinetic experiments were carried out at pH 8.2 and 30 °C in the presence of 1 mM NAD and 0.05–1.4 M PPA. A linear dependence of the acylation rate versus PPA concentration was observed up to 1.4 M, irrespective of the mutated MSDH. For R124L and R301L MSDHs, k2 values of 110 and 114 M⁻¹s⁻¹ were estimated which are ~2.5-fold higher than the k2 value of the wild-type MSDH (Table 4). Moreover, the kobs value determined at 0.05 M PPA for R124L MSDH is 2-fold higher than the kcat value (i.e. 5.5 s⁻¹ vs 2.8 s⁻¹). The kobs value for R124L MSDH supports a rate-limiting step that occurs after the hydride transfer, while no definitive conclusion can be made for R301L MSDH due to the fact that the kcat value of 3 s⁻¹ is likely to be an underestimate.

The fact that in the absence of CoA, the kobs was higher than the kcat (at least for R124L MSDH) did not exclude CoA binding to the mutated MSDHs, and therefore a kinetic effect of CoA on the acylation step. To evaluate this assumption, fast kinetic experiments were carried out on R124L MSDH in the presence of 1 mM NAD and 250 µM CoA, with PPA concentrations ranging from 0–0.54 M. Under these experimental conditions, a Kapp of 0.18 M for PPA and a kobs max of 110 s⁻¹ were determined (Supplemental data S4). Therefore, binding of CoA leads to a 5.5–fold increase of the second-order rate constant kobs max/Kapp when compared to the k2 value determined in the absence of CoA. Similar experiments were carried out in the presence of 1 mM NAD and 0.54 M PPA with CoA concentrations ranging from 0–400 µM. A Kapp of 90 µM for CoA was determined which is
in the range of that found for the wild-type MSDH (35 µM).

The \( k_{\text{obs}} \) value determined at 0.05 M of PPA is ~5-fold higher than the \( k_{\text{obs}} \) determined in the absence of CoA and 10-fold higher than the \( k_{\text{cat}} \) value (i.e. 29 s\(^{-1}\) vs ~3 s\(^{-1}\)). This latter result confirmed that the rate-limiting step takes place after hydride transfer as already shown for the wild-type MSDH. For the R301L MSDH, similar experimental conditions were used except the concentration range of PPA was 0–0.35 M, yielding a \( K_{\text{app}} \) of 0.16 M for PPA and a \( k_{\text{obs}} \) of 117 s\(^{-1}\) (Supplemental data S4). As observed for R124L MSDH, binding of CoA led to a 6.5-fold increase in the second-order rate constant \( k_{\text{obs max}}/K_{\text{app}} \) relative to the \( k_{2} \) value determined in the absence of CoA.

Additionally, the rate constants for NADH dissociation from the thioacylenzyme-NADH complex were determined for R124L and R301L MSDHs. The experiments were performed at pH 8.2 and 30 °C under pre-steady-state conditions and in the presence of 1 mM NAD and 0.5 M PPA, but in the absence of CoA. Apparent rate constants of 12 s\(^{-1}\) and 8.4 s\(^{-1}\) were assigned to NADH release for R124L and R301L MSDHs, respectively (curves not shown), in the range of that determined for the wild-type MSDH. These rates are higher than the \( k_{\text{cat}} \) values at least for R124L MSDH (Table 3). Thus, it can be concluded that the rate-limiting step for R124L MSDH and probably also for R301L MSDH, is associated with the transthioesterification process. This finding accords with the fact that the \( k_{\text{cat}} \) values for R124L and R301L MSDHs with MMSA as substrate, for which the rate-limiting step is likely associated with the \( \beta \)-decarboxylation process, are at least 20-fold lower compared with those determined with PPA. The fact that the rate-limiting step for wild-type MSDH with PPA is associated with NADH release (12.6 s\(^{-1}\)) supports an intrinsic rate of transthioesterification which is at least 4-fold higher than that of R124L MSDH.

Finally, for the R124L MSDH, the pH-dependence of the acylation rate constant was determined in the presence of 1 mM NAD, 0.5 M PPA and 500 µM CoA. As already described for the wild-type MSDH, the formation of NADH was measured by monitoring the FRET signal using a 395 nm cut-off filter. The pH\(-k_{\text{ac}}\) curve exhibited an increasing bisigmoidal profile that can be related to the contribution of two ionizable groups, one of pK\(_{\text{app}}\) 5.7 that must be deprotonated for acylation and one of pK\(_{\text{app}}\) 7.5 whose deprotonation increased the acylation rate by 1.5-fold with rate constants \( k_{\text{ac i}} \) of 67 s\(^{-1}\) and \( k_{\text{ac max}} \) of 92 s\(^{-1}\), respectively (Figure 6). The pH-dependence of the steady-state rate constant was determined at 30 °C in the presence of 1 mM NAD, 0.05 M PPA and 500 µM CoA. The pH-\( k_{\text{ss}} \) curve exhibited a monosigmoidal profile showing dependence on the contribution of one ionizable group of pK\(_{\text{app}}\) 6.8, and a \( k_{\text{ss max}} \) of 1.3 s\(^{-1}\) (Figure 7). Differences in buffer composition and ionic strength likely explain why the \( k_{\text{ss max}} \) is half of the \( k_{\text{cat}} \) value i.e. 2.8 s\(^{-1}\).

**DISCUSSION**

The kinetic mechanism of the CoA-dependent ALDH family to which MSDHs belong, was shown to be of ping-pong type with NADH release preceding transthioesterification (5,6). However, the MSDH-catalyzed reaction also includes a supplementary \( \beta \)-decarboxylation step that occurs before transthioesterification. Our recent mechanistic characterization of the wild-type MSDH from *B. subtilis* showed that the \( \beta \)-decarboxylation process is rate-limiting and occurs with formation of bicarbonate. As discussed earlier, sequence alignments and three-dimensional structure comparisons suggested that the two invariant residues Arg124 and Arg301 in MSDHs, contributed to the binding of MMSA through electrostatic interaction between the carboxylate and the guanidinium groups. This assumption is supported by the kinetic parameters of the acylation step for R301L, R124L and R124L/R301L MSDHs. Indeed, no saturating kinetic effect with respect to MMSA was observed up to 12–16 mM, in contrast to the wild-type MSDH. The second-order rate constant \( k_{2} \) is decreased by a factor of 55, 62 and at least 512 for R124L, R301L and R124L/R301L MSDHs, respectively. The magnitude of these decreases is 3- to 30-fold higher than that described for R124L GAPN (28). In the case of MSDH, the decrease of \( k_{2} \) likely includes contributions from both \( K_{\text{app}} \) and \( k_{\text{obs max}} \). Therefore, Arg124 and Arg301 participate not only in MMSA binding via a stabilizing electrostatic interaction with the carboxylate but also likely in positioning MMSA efficiently relative to Cys302 within the ternary complex MSDH-NAD-MMSA. From an evolutionary point of view, it is interesting to note that such a stabilization mode is not unique.
within the ALDH superfamily. Indeed, in the succinate semialdehyde dehydrogenase (SSADH), a member of the hydrolytic ALDH family, the stabilization of the carboxylate group of the substrate is mainly achieved through hydrogen bonds with two arginine (Arg213 and Arg334) residues (29). Although differing in position in primary structures, superimposition of the GAPN, MSDH and SSADH active sites shows that Arg213 and Arg334 are structurally and functionally equivalents to Arg124 and Arg301, respectively (not shown).

With the PPA substrate, no kinetic role of Arg124 and Arg301 in the acylation process was expected due to the absence of the carboxylate group. This assumption is supported by the values of the second-order rate constant \( k_2 \) of the acylation step determined in the absence of CoA, which are similar for the wild-type and the mutated MSDHs (i.e. 36, 110 and 114 M\(^{-1}\)s\(^{-1}\), respectively) and which are \(-10^2\)-fold lower compared to that determined for the wild-type MSDH with MMSA as substrate. Much more importantly, the \( k_{obs} \) value determined at 50 mM PPA (i.e. a saturating concentration under steady-state conditions) was 2.5-fold lower than the \( k_{cat} \) value. This result suggested that CoA binds to the wild-type MSDH prior to the hydride transfer. Favouring this assumption is the fact that the \( k_2 \) value for the acylation step determined in the presence of saturating concentration of CoA is 72-fold higher than in the absence of CoA. The increase of the \( k_2 \) value is mainly due to a \( K_{app} \) effect for PPA, as it is decreased by a factor of at least 25. This result suggests a higher affinity of the wild-type MSDH for PPA due to CoA binding. When CoA is bound, the \( K_{app} \) of PPA is only 40-fold higher than that for MMSA in the acylation step. The stronger affinity with MMSA is likely due to stabilizing interactions between the carboxylate of MMSA and both Arg124 and Arg301.

The R124L and R301L MSDHs also exhibited an increase in \( k_2 \) by factors of 5.5-fold and 6.5-fold, respectively, in the presence of 250 \( \mu \)M CoA. Again, as for the wild-type MSDH, the increase of the \( k_2 \) value is mainly due to a \( K_{app} \) effect for PPA, which suggests a higher affinity of the mutated MSDHs for PPA. These results, taken together, raise interesting questions as to the nature of the molecular/structural factors that favour the increased affinity of the wild-type, R124L and R301L MSDHs-NAD complexes for PPA in the presence of CoA. The only information currently available on this issue is that the thiol group of CoA plays no role because the kinetics of the acylation step are similar when desulfo-CoA is used instead of CoA. Substituting Leu for Arg124 led to an increase of the \( K_{app} \) and \( K_M \) values for CoA by factors of 3 and 4, respectively. Considering the 4.5-fold decrease of the \( k_{cat} \) value, the catalytic efficiency of the thioester exchange with CoA (~ 8.5 \( \times \) 10\(^5\) M\(^{-1}\)s\(^{-1}\)) is at least 17-fold decreased (~ 1.5 \( \times \) 10\(^5\) M\(^{-1}\)s\(^{-1}\) for the wild-type MSDH). The Arg124 probably favours an efficient binding of CoA within the deacylation complex. This is likely also the case for Arg301 whose substitution by Leu leads to an even more significant increase of the \( K_M \) value for CoA.

In contrast to PPA, when MMSA was used as substrate, no kinetic effect on the acylation step was observed for the wild-type and R301L MSDHs when CoA was added. Therefore, two alternatives are possible: either CoA binds but has no kinetic effect on the rate of the acylation step or CoA does not bind. Further information on CoA binding to the binary complex MSDH-NAD comes from our earlier studies of the lag phase in enzymatic turnover (25). Indeed, we have shown that formation of the MSDH-NAD binary complex elicits a slow conformational reorganization within the active site of the wild-type MSDH. The lag phase, which is eliminated when the wild-type and R301L MSDHs are preincubated with NAD, also disappears for R124L and R124L/R301L MSDHs but only when CoA is also added. This supports CoA binding at minimum to the R124L and R124L/R301L MSDH-NAD complexes. Thus, for mutants where Arg124 is substituted by Leu, the lag phase is also a good probe of the CoA binding to the enzyme-NAD complex. The reasons why R124L mutants behave differently than R301L and wild-type MSDHs remain to be explained.

Together, the kinetic data obtained in the present study favor the existence of two distinct binding sites for CoA and NAD in contrast to the suggestion made in our previous paper which was based on the hypothesis that the early release of NADH (i.e. before transthioesterification) occurs because the NADH binding site partially overlaps the CoA binding site.

The fact that with PPA as substrate, CoA binds to wild-type MSDH in the acylation step gave us the opportunity to measure the \( pK_{app} \) of the thiol group of CoA in the acylation step. In the absence of CoA, the obtained \( k_{ac} \) versus–pH
curve with PPA fits to a monosigmoidal profile, yielding a $pK_{\text{app}}$ of $\sim 6$. This value is similar to that previously found for the wild-type MSDH and the quintuple Cys$\rightarrow$Ala mutant, in which Ala was substituted for all the Cys residues, except for Cys302, with MMSA as substrate (25). Therefore, as already interpreted, the $pK_{\text{app}}$ of $\sim 6$ corresponds to the $pK_{\text{app}}$ of Cys302. In the presence of saturating concentrations of CoA, the pH-$k_{\text{ac}}$ curve exhibits an increasing double sigmoidal profile with two $pK_{\text{app}}$ values of 6.5 and 7.9 (Figure 4). The double sigmoidal profile is therefore representative of two species of Cys302 that participate in the acylation process i.e. Cys302-S$\rightarrow$/CoA-SH with a $k_{\text{ac}}$ of 100 s$^{-1}$ and Cys302-S$\rightarrow$/CoA-$S\rightarrow$ with a $k_{\text{ac}}$ of 330 s$^{-1}$. Consequently, the $pK_{\text{app}}$ of 6.5 corresponds to Cys302, while that of 7.9 can be assigned to the thiol group of CoA. Indeed, when desulfo-CoA was used instead of CoA (i.e. no CoA-SH species was present), a monosigmoidal profile is observed with a $pK_{\text{app}}$ of 6.5 (Figure 4).

As NADH dissociation from the acylation complex was shown to be rate-limiting, it was not possible to determine the $pK_{\text{app}}$ of the CoA within the deacylation complex. The picture is clearly different for at least R124L MSDH for which the rate-limiting step is now associated with transthioesterification. From the pH-$k_{\text{ac}}$ curve obtained for R124L MSDH in the presence of CoA (Figure 6), two $pK_{\text{app}}$ of 5.7 and 7.5 were extracted, which can be assigned to Cys302 and CoA, as already proposed for the wild-type MSDH. The fact that a monosigmoidal profile was obtained for the pH-$k_{\text{ss}}$ curve (Figure 7) with a $pK_{\text{app}}$ of 6.8 suggests a similar $pK_{\text{app}}$ for both Cys302 and CoA. Alternatively, the $pK_{\text{app}}$ of 6.8 corresponds only to that of CoA. In any case, the $pK_{\text{app}}$ values shows that the CoA is more activated in the deacylation than in the acylation complex, consistent with the requirement for it to efficiently attack the thioacylenzyme intermediate. Presumably, the change in $pK_{\text{app}}$ between the two complexes arises from differences in the local environment or precise positioning of the thiol of CoA. In addition, the $k_2$ ($k_{\text{cat}}/K_M$) values for the MSDH thioester exchange with CoA are $\geq 10^4$, and $\approx 10^3$-fold higher for the wild-type and R124L MSDHs, respectively when compared to the second-rate constant $k_2$ for the attack of the mercaptoethanol anion (which has a $pK_{\text{app}}$ value of 9.6 (30) similar to that of CoA), on a non activated thiol ester i.e. $12\, M^{-1}\, s^{-1}$ (31). Among the different factors that can explain such differences, a major one should be CoA binding to MSDH that likely optimizes the positioning of CoA relative to the thioacylenzyme intermediate and thus favours a significant decrease of its $pK_{\text{app}}$.

In conclusion, Arg301 and Arg124 are essential in the binding of MMSA via stabilizing interactions with the carboxylate group. The use of PPA has permitted to show that the CoA also binds to the acylation complex and therefore to a site distinct from that of NAD. The catalytic efficiency of the MSDH thioester exchange with CoA is at least $10^4$-fold increased compared to the second-rate constant $k_2$ for the attack of the mercaptoethanol anion on a non activated thiol ester. This difference in reactivity is the consequence of the binding of CoA to MSDH which likely optimizes the positioning of CoA relative to the thioacylenzyme intermediate and consequently favors a significant decrease of approx. 3 units of the $pK_{\text{app}}$ of the thiol of CoA. The results obtained in the present study leave some questions opened. For example, as the NAD and CoA binding sites do not overlap, the CoA-binding site remains to be characterized. In this context, the resolution of the three-dimensional structure of a ternary MSDH-NAD-CoA complex would be informative.

**REFERENCES**


**FOOTNOTES**

We gratefully thank S. Bouterin for her very efficient technical help, and Pr. C. Gerardin for her help to synthesize desulfo-CoA. We also thank Pr. K. Weissman for careful reading of the manuscript. C. S-C. was supported by the French Research Ministry. This work was supported in part by the CNRS, the University of Nancy I, the Institut Fédératif de Recherche 111 Bioingénierie and local funds from the Région Lorraine.

3 Supported by the French Research Ministry
Abbreviations used are: ALDH, aldehyde dehydrogenase; D-G3P, D-glyceraldehyde 3-phosphate; GAPN, non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; MMSA, methylmalonate semialdehyde; MSDH, methylmalonate semialdehyde dehydrogenase; PPA, propionaldehyde; SSADH, succinate semialdehyde dehydrogenase.
Scheme 1 Schematic representation of MSDH-catalyzed reaction with MMSA as substrate (adapted from (25)). When PPA is used as substrate, CoA attacks the same thiopropionylenzyme intermediate. The acylation step comprises all the events up to the hydride transfer while the deacylation step includes release of NADH up to release of thiopropionylCoA.
Kinetic parameters were deduced from non-linear least square regression of experimental data sets, according to the Michaelis-Menten equation. All $K_M$ values were determined at saturating concentrations of the other substrates and $k_{cat}$ values are expressed per active subunit (i.e. two active subunits per tetramer). The steady-state initial rates of the reaction of mutated MSDHs were measured at 30 °C in 50 mM potassium phosphate buffer, (pH 8.2), under similar conditions as those for the wild-type MSDH. Mutated MSDHs were pre-incubated with 2 mM NAD or with 2 mM NAD and 500 µM CoA at 30°C, prior to making the kinetic measurements.

(a) from (25)

† After dilution of the preincubated enzymes, the remaining NAD (12 µM) and CoA (3 µM) concentrations were taken into account for determination of the $K_M$ values.

‡ For all MSDHs, the rate-limiting step remains associated with the so-called “deacylation” step (i.e. all the steps occurring after hydride transfer)

†† Due to lack of saturation with substrates and very low steady-state initial rates (< 10^{-2} s^{-1}), it was not possible to determine the $k_{cat}$ and $K_M$ values.

Table 1: Kinetic parameters of the reactions catalyzed by wild-type and mutated MSDHs under steady-state conditions with MMSA as substrate.

<table>
<thead>
<tr>
<th></th>
<th>$K_M$ MMSA (mM)</th>
<th>$K_M$ CoA$^+$ (µM)</th>
<th>$K_M$ NAD$^+$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)$^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (a)</td>
<td>0.06 ± 0.01</td>
<td>120 ± 20</td>
<td>2.30 ± 0.06</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>R124L</td>
<td>0.006 ± 0.001</td>
<td>570 ± 70</td>
<td>0.06 ± 0.01</td>
<td>0.060 ± 0.01</td>
</tr>
<tr>
<td>R301L</td>
<td>0.023 ± 0.005</td>
<td>620 ± 80</td>
<td>0.12 ± 0.01</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>R124L/R301L</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>&lt; 10^{-2}$^\dagger$</td>
</tr>
</tbody>
</table>
Table 2 : Determination of the kinetic parameters for the acylation step for R124L, R301L and R124L/R301L MSDHs under pre-steady-state conditions with MMSA as substrate.

Pre-steady-state measurements were performed at 30 °C in 50 mM potassium phosphate buffer, (pH 8.2). Mutated MSDHs were pre-incubated with 2 mM NAD (R301L MSDH) or with 2 mM NAD and 500 µM CoA (R124L and R124L/R301L MSDHs) at 30 °C prior to making the kinetic measurements. The $k_2$ values of the acylation step were obtained by linear regression of $k_{obs}$ determined under sub-saturating concentration of substrate MMSA (up to 16 mM). For the wild-type MSDH, the second-order rate constant $k_2$ was obtained by dividing $k_{obs max}$ by $K_{app}$.

(a) from (25)

<table>
<thead>
<tr>
<th></th>
<th>$K_{app}$ MMSA (mM)</th>
<th>$k_{obs max}$ (s$^{-1}$) MMSA</th>
<th>$k_2$ (M$^{-1}$. s$^{-1}$) MMSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (a)</td>
<td>3.5 ± 1.0</td>
<td>1200 ± 180</td>
<td>3.4x10$^5$</td>
</tr>
<tr>
<td>R124L</td>
<td>&gt; 15</td>
<td>/</td>
<td>6x10$^3$</td>
</tr>
<tr>
<td>R301L</td>
<td>&gt; 15</td>
<td>/</td>
<td>5.4x10$^5$</td>
</tr>
<tr>
<td>R124L/R301L</td>
<td>&gt; 15</td>
<td>/</td>
<td>6.5x10$^2$</td>
</tr>
</tbody>
</table>

Table 3 : Kinetic parameters for the reactions catalyzed by the wild-type and mutated MSDHs under steady-state conditions with PPA as substrate.

Kinetic parameters were deduced from non-linear least square regression of experimental data sets, according to the Michaelis-Menten equation. All $K_M$ values were determined at saturating concentrations of the other substrates and $k_{cat}$ values are expressed per active subunit (i.e. two active subunits per tetramer). The steady-state initial rates of the reaction of wild-type and mutated MSDHs were measured at 30 °C in 50 mM potassium phosphate buffer, (pH 8.2). Wild-type and mutated MSDHs were pre-incubated with 2 mM NAD at 30 °C before kinetic measurements. nd : not determined.

‡ For all MSDHs, the rate-limiting step remains associated with the so-called “deacylation” step (i.e. all the steps occurring after hydride transfer).

+ After dilution of the preincubated enzyme, the remaining NAD concentration (2 µM) was taken into account for determination of the $K_M$ value.

† This value is only an estimate, since the kinetic parameters can not be determined with accuracy due to lack of saturation with CoA.

<table>
<thead>
<tr>
<th></th>
<th>$K_M$ PPA (mM)</th>
<th>$K_M$ CoA (µM)</th>
<th>$K_M$ NAD$^+$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)$^+$</th>
<th>$k_{cat}/K_M$ CoA (M$^{-1}$.s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>9.8 ± 0.3</td>
<td>85 ± 10</td>
<td>33 ± 13</td>
<td>12.6 ± 0.2</td>
<td>1.5x10$^5$</td>
</tr>
<tr>
<td>R124L</td>
<td>9.3 ± 1.1</td>
<td>330 ± 35</td>
<td>38 ± 1</td>
<td>2.8 ± 0.4</td>
<td>8.5x10$^3$</td>
</tr>
<tr>
<td>R301L</td>
<td>nd</td>
<td>&gt; 1000</td>
<td>nd</td>
<td>&gt; 3</td>
<td>3.0x10$^3$†</td>
</tr>
</tbody>
</table>
Table 4: Determination of the kinetic parameters of the acylation step for the reactions catalyzed by wild-type, R124L and R301L MSDHs with PPA as substrate.

Pre-steady-state measurements were performed at 30 °C in 50 mM potassium phosphate buffer, (pH 8.2) in the absence or the presence of CoA. Wild-type and mutated MSDHs were pre-incubated with 2 mM NAD at 30 °C prior to making the kinetic measurements. In the absence of CoA, the appearance of NADH was monitored by following the change in absorbance at 340 nm. The $k_2$ values were obtained by linear regression of $k_{obs}$ measured at sub-saturating concentrations of PPA up to 1.4 M. For the wild-type MSDH, the second-order rate constant $k_2$ was obtained from estimations of the $K_{app}$ and the $k_{obs max}$ values. In the presence of 500 µM CoA (wild-type) or 250 µM CoA (R124L and R301L MSDHs), formation of NADH was measured by monitoring the FRET signal using a 395 nm cut-off filter, upon selective excitation of Trp residues at 295 nm. The concentration of PPA was varied from 0 to 0.54 M and 0 to 0.35 M for R124L and R301L mutants, respectively (see Supplemental data S4).

<table>
<thead>
<tr>
<th></th>
<th>$K_{app}$ PPA (M)</th>
<th>$k_{obs max}$ (s$^{-1}$)</th>
<th>$k_2$ (M$^{-1}$s$^{-1}$)</th>
<th>$K_{app}$ PPA (M)</th>
<th>$k_{obs max}$ (s$^{-1}$)</th>
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<tr>
<td></td>
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</tr>
<tr>
<td>Wild-type</td>
<td>2.6 ± 0.6</td>
<td>120 ± 20</td>
<td>36</td>
<td>0.15 ± 0.02</td>
<td>340 ± 20</td>
</tr>
<tr>
<td>R124L</td>
<td>&gt; 3</td>
<td>/</td>
<td>110</td>
<td>0.18 ± 0.03</td>
<td>110 ± 7</td>
</tr>
<tr>
<td>R301L</td>
<td>&gt; 3</td>
<td>/</td>
<td>114</td>
<td>0.16 ± 0.05</td>
<td>117 ± 16</td>
</tr>
</tbody>
</table>
Figures Legends

Fig. 1. Sequence alignment of the regions around the conserved catalytic Cys302 and Arg residues in MSDHs and comparison with other ALDHs. The sequence alignment comprises some MSDHs whose activity has been demonstrated. Sequence alignment was performed with the BioEdit software and the numbering of amino acid residues is according to Wang and Weiner (8). The conserved catalytic Cys302, and the Arg124 and Arg301 residues which are only conserved in MSDH and GAPN sequences are highlighted. Sequences of 19 GAPNs and 40 hydrolytic and CoA-dependent ALDHs (excluding GAPNs) from the EMBL, Swiss-Prot, GenBank™, and Pir databases were aligned to determine the consensus sequences.

Fig. 2. Determination of the catalytic parameters of the acylation step for R124L, R301L and R124L/R301L MSDH-catalyzed reactions under pre-steady-state conditions with MMSA as substrate. The appearance of NADH was monitored at 340 nm on a SX18MV-R stopped-flow apparatus (Applied PhotoPhysics) at 30 °C. The final concentration of MSDH was 16 µM (expressed in subunit) with the MMSA concentration ranging from 1 to 16 mM. For each substrate concentration, experimental data were analyzed by nonlinear regression using the SX18MV-R software package to obtain $k_{\text{obs}}$. As saturation with MMSA was not observed irrespective of the mutated MSDHs, $k_{\text{obs}}$ were extracted from the resulting linear equations. Key: R124L MSDH (●, solid line); R301L MSDH (▲, dashed line), R124L/R301L MSDH (■, solid line) and wild-type MSDH (♦, solid line, from [25]). The inset details the results obtained on the three mutated MSDHs.

Fig. 3. Determination of the catalytic parameters for the acylation step of wild-type MSDH in the presence of PPA, under pre-steady-state conditions. The appearance of NADH was selectively monitored using fluorescence resonance energy transfer (FRET) on a SX18MV-R stopped-flow apparatus (Applied PhotoPhysics) at 30 °C. One syringe filled with 16 µM wild-type MSDH (expressed in subunit), 1 mM NAD and 500 µM CoA (final concentrations), while the other one contained PPA at various concentrations. Following selective excitation of Trp residues at 295 nm, the fluorescence emission of NADH was measured using a 395 nm cut-off filter. Rate constants $k_{\text{obs}}$ were fit to Equation 1, which gave a $k_{\text{obs max}}$ of $340 \pm 20$ s$^{-1}$ and a $K_{\text{app}}$ for PPA of $0.15 \pm 0.02$ M. The inset shows the results obtained in the absence of CoA or desulfo-CoA.

Fig. 4. pH-dependence of the acylation rate $k_{\text{ac}}$ for the wild-type MSDH-catalyzed reaction with PPA as substrate. Pre-steady-state data were collected at 30 °C on a SX18MV-R stopped-flow apparatus (Applied PhotoPhysics) using the 20 µL cell, by rapidly mixing 16 µM wild-type MSDH, 1 mM NAD, 0.5 M PPA and 500 µM CoA (final concentrations) over a pH-range of 5.00–8.75 in buffer A. At pHs lower than 5, MSDH was not stable. Following selective excitation of Trp residues at 295 nm, formation of NADH was measured by monitoring the FRET signal using a 395 nm cut-off filter. Each individual point is the average of at least 6 measurements with an error < 5 %. Experimental data (●) were fit by non-linear least-square regression analysis against a two-pKa model which gave p$K_{\text{app}}$s of $6.5 \pm 0.1$ and $7.9 \pm 0.3$ with rate constants $k_{\text{ac i}}$ of $176 \pm 28$ s$^{-1}$ and $k_{\text{ac max}}$ of $330 \pm 16$ s$^{-1}$, respectively (solid line). The data do not fit to a one-pKa model (dotted line). The inset shows the results obtained in the presence of 250 µM desulfo-CoA. Experimental data were fit by non-linear least square regression analysis against a one-pKa model which gave a p$K_{\text{app}}$ of $6.5 \pm 0.1$ with rate constant $k_{\text{ac}}$ of $162 \pm 2$ s$^{-1}$ (solid line).
**Fig. 5.** pH-dependence of the steady-state rate constant $k_{ss}$ for wild-type MSDH with PPA as substrate. The pH-dependence of $k_{ss}$ was studied at 30 °C over the pH range 5.0-9.0 in buffer A. The remaining experimental conditions were as follows: 1 mM NAD, 0.05 M PPA and 500 µM CoA. Experiments were done twice with experimental errors on the individual points ≤ 5%. Data (●) were fit by non-linear least square regression analysis against a two-$pK_{app}$ model which yielded $pK_{app}$s of 6.5 ± 0.1 and 7.8 ± 0.1, and rate constants $k_{ss}$ of 6.0 ± 0.7 s$^{-1}$ and $k_{ss}$ max of 18 ± 0.2 s$^{-1}$, respectively (solid line). The data do not fit to a one-$pK_{a}$ model (dotted line).

**Fig. 6.** pH-dependence of the acylation rate constant $k_{ac}$ for the R124L MSDH-catalyzed reaction with PPA as substrate. Pre-steady-state data were collected at 30 °C on a SX18MV-R stopped-flow apparatus (Applied PhotoPhysics) using the 20 µL cell, by rapidly mixing 16 µM R124L MSDH (expressed in subunit), 1 mM NAD, 0.5 M PPA and 500 µM CoA (final concentrations) over a pH-range of 5.50–8.75 in buffer A. Following excitation at 295 nm, formation of NADH was measured by monitoring the fluorescence emission using a 395 nm cut-off filter. Each individual point is the average of at least 6 measurements with an error < 5 %. Experimental data (●) were fit by non-linear least square regression analysis against a two-$pK_{a}$ model which gave $pK_{a}$ of 5.7 ± 0.1 and 7.5 ± 0.2, and rate constants $k_{ac}$ of 67 ± 4 s$^{-1}$ and $k_{ac}$ max of 92 ± 2 s$^{-1}$, respectively (solid line). The data do not fit to a one-$pK_{a}$ model (dotted line).

**Fig. 7.** pH-dependence of the steady-state rate constant $k_{ss}$ for the R124L MSDH-catalyzed reaction with PPA as substrate. The pH-dependence of $k_{ss}$ was studied at 30 °C over the pH range 5.00-8.75 in buffer A. The remaining experimental conditions were as follows: 1 mM NAD, 0.05 M PPA and 500 µM CoA. Experiments were done twice with experimental errors on the individual points ≤ 5%. Data (●) were fit by non-linear least square regression analysis against a single $pK_{app}$ model which yielded a $pK_{app}$ of 6.8 ± 0.1 and $k_{ss}$ of 1.3 ± 0.1 s$^{-1}$ (solid line).
Figure 1

<table>
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</table>

Figure 2

![Graph showing enzymatic activity](chart.png)

Insert graph showing enzymatic activity with various [MMSA] (mM) and $k_{obs}$ (s$^{-1}$) values.
Figure 3

![Graph showing the relationship between [PPA] (M) and $k_{ob}$ (s$^{-1}$).](image)

Figure 4

![Graph showing the relationship between pH and $k_{ac}$ (s$^{-1}$).](image)
Figure 5

Figure 6
Figure 7

![Graph showing pH and $k_s$ relationship](http://www.jbc.org/)

- pH range: 5 to 9
- $k_s$ range: 0.0 to 1.5
- The graph illustrates the increase in $k_s$ with respect to pH, indicating a non-linear relationship.

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