Mechanistic characterization of the MSDH (methylmalonate semialdehyde dehydrogenase) from *Bacillus subtilis*

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Homotetrameric MSDH (methylmalonate semialdehyde dehydrogenase) from *Bacillus subtilis* catalyses the NAD-dependent oxidation of MMSA (methylmalonate semialdehyde) and MSA (malonate semialdehyde) into PPCoA (propionyl-CoA) and acetyl-CoA respectively via a two-step mechanism. In the present study, a detailed mechanistic characterization of the MSDH-catalysed reaction has been carried out. The results suggest that NAD binding elicits a structural imprinting of the apoenzyme, which explains the marked lag-phase observed in the activity assay. The enzyme also exhibits a half-of-the-sites reactivity, with two subunits being active per tetramer. This result correlates well with the presence of two populations of catalytic Cys³⁰² in both the apo- and holo-enzymes. Binding of NAD causes a decrease in reactivity of the two Cys³⁰² residues belonging to the two active subunits and a pK_{app} shift from approx. 8.8 to 8.0. A study of the

rate of acylation as a function of pH revealed a decrease in the pK_{app} of the two active Cys³⁰² residues to approx. 5.5. Taken together, these results support a sequential Cys³⁰² activation process with a pK_{app} shift from approx. 8.8 in the apo-form to 8.0 in the binary complex and finally to approx. 5.5 in the ternary complex. The rate-limiting step is associated with the β -decarboxylation process which occurs on the thioacyl-enzyme intermediate after NADH release and before transthiolysis. These data also indicate that bicarbonate, the formation of which is enzyme-catalysed, is the end-product of the reaction.

Key words: bicarbonate, CoA-dependent aldehyde dehydrogenase (ALDH), cysteine activation, β -decarboxylation, half-of-the-sites reactivity, lag-phase.

INTRODUCTION

Two structurally unrelated families of NAD(P)-dependent ALDHs (aldehyde dehydrogenases) catalyse the oxidation of aldehydes into acidic compounds via a two-step chemical mechanism, first with an acylation step common to both families, and then a deacylation step which differs in the nature of the acyl acceptor. The acylation step involves the nucleophilic attack of the catalytic cysteine residue on the aldehydic function followed by the hydride transfer that leads to formation of a thioacyl-enzyme intermediate and NAD(P)H. In phosphorylating ALDHs, inorganic phosphate acts as the acyl acceptor, thus leading to formation of a phospho-ester. This, for example, is the case in GAPDH (glyceraldehyde-3-phosphate dehydrogenase). By contrast, in the non-phosphorylating ALDHs, the acyl-enzyme intermediate undergoes a nucleophilic attack by a water or CoA molecule, thus leading to non-activated or CoA-activated acids respectively (Scheme 1). Numerous crystal structures of homotetrameric and homodimeric non-phosphorylating CoA-independent ALDHs have already been solved and essentially show identical structural features at the secondary, tertiary and quaternary levels [1–3]. Moreover, mechanistic aspects were studied extensively and several invariant residues were shown to be critical for the chemical mechanism of non-phosphorylating CoA-independent ALDHs [4–7]. Subsequently, evidence has been provided for the chemical activation of the catalytic Cys³⁰² upon cofactor binding to non-phosphorylating GAPN (non-phosphorylating GAPDH) from *Streptococcus mutans* [8]. Moreover, additional studies of this GAPN have highlighted the essential roles of (i) an oxyanion-hole composed of the side-chain of the invariant Asn¹⁶⁹ residue and the Cys³⁰² NH-group which permits

an efficient hydride transfer without base-catalyst assistance [9] and (ii) the Glu²⁶⁸ residue in the rate-limiting hydrolytic step through activation and orientation of the attacking water molecule [10].

By contrast, little information regarding the mechanistic and/or structural aspects of CoA-dependent ALDHs is available so far, excepting the crystal structure of a bifunctional enzyme 4-hydroxy 2-ketovaleate aldolase/acylating acetaldehyde dehydrogenase described recently [11]. However, from a structural viewpoint, this CoA-dependent dehydrogenase does not belong to the non-phosphorylating ALDH family, but to the phosphorylating GAPDH family.

In the present paper, a detailed study of the enzymatic properties of the MSDH [MMSA (methylmalonate semialdehyde) dehydrogenase] from *Bacillus subtilis*, which is one of the few CoA-dependent ALDHs [12,13], has been undertaken. MSDH from *B. subtilis* is a homotetrameric enzyme putatively involved in *myo*-inositol catabolism [14]. It catalyses the NAD-dependent oxidation of MMSA and MSA (malonate semialdehyde) into PPCoA (propionyl-CoA) and acetyl-CoA respectively (Scheme 2). Therefore the oxidation process catalysed by MSDH from *B. subtilis* includes a β -decarboxylation step, which is a specific feature when compared with the chemical mechanism of the non-phosphorylating CoA-independent and CoA-dependent ALDHs described so far. As expected, sequence alignment reveals conservation of the catalytic Cys³⁰² and Asn¹⁶⁹ residues but the lack of Glu²⁶⁸, which is consistent with the non-efficient hydrolytic activity exhibited by MSDHs.

The results obtained in the present study suggest that NAD binding elicits a slow conformational rearrangement [(imprinting), where conformational rearrangement can include as little as

Abbreviations used: ALDH, aldehyde dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAPN, non-phosphorylating GAPDH; IAM, iodoacetamide; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; MMSA, methylmalonate semialdehyde; MSA, malonate semialdehyde; MSDH, MMSA dehydrogenase; 2PDS, 2,2'-dipyridyl disulphide; PEP, phosphoenolpyruvate; PEPC, PEP carboxylase; PPCoA, propionyl-CoA.

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Scheme 1 Schematic representation of the catalytic mechanism of non-phosphorylating ALDHs

Scheme 2 Schematic representation of MSDH-catalysed reactions as proposed from kinetic studies (see the Results and Discussion sections)

R represents a CH_3 group in MMSA and PPCoA, whereas it represents a hydrogen atom in MSA and acetyl-CoA.

a reorientation of the Cys³⁰² residue and repositioning of either the NAD or the quenched Trp⁴⁶⁸, located at the dimer interface, or both] in the apo-structure of the MSDH from *B. subtilis*, which explains the marked lag-phase observed in the activity assay. The enzyme also exhibits a half-of-the-sites reactivity. Only two subunits per tetramer display activity, which correlates well with the presence of two populations of the catalytic Cys³⁰² residue in both the apo- and holo-enzymes. In addition, kinetic analysis of the catalytic mechanism showed that the rate-limiting step is associated with the β -decarboxylation process which occurs on the thioacylzyme after NADH release and before transthiostereification. The results and interpretation are discussed in relation to what is known about the catalytic mechanism and the three-dimensional structure of the non-phosphorylating ALDHs, in particular GAPN.

EXPERIMENTAL

Materials

NAD was from Roche (Mannheim, Germany). CoA, propionaldehyde, pyruvate, PEP (phosphoenolpyruvate), LDH (lactate dehydrogenase) and MDH (malate dehydrogenase) were from Sigma (St. Louis, MO, U.S.A.), and PEPC (PEP carboxylase) was from Fluka (Buchs, Switzerland).

MMSA was synthesized as described by Kupiecki and Coon [15] and MSA was prepared from ethyl ester diethyl acetal of MSA (Acros Organics, Noisy-le-Sec, France). Both substrates were enzymatically titrated with MSDH.

Site-directed mutagenesis, production and purification of wild-type and mutant MSDH from *B. subtilis*

Site-directed mutagenesis was performed using the Quik-change site-directed mutagenesis kit (Stratagene). Wild-type, QCys (C49A/C176A/C305A/C369A/C403A), W28F, W76F, W177F, W397F and W468F MSDHs were produced and purified by using a procedure already described [16]. Wild-type and mutant MSDHs were isolated as apo-forms. Enzyme concentrations were determined spectrophotometrically by using molar absorption coefficients of $204\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$ at 280 nm for apo-(wild-type) and QCys MSDHs, $180\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$ for W28F MSDH, $164\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$ for W76F, W177F and W397F MSDHs and $172\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$ for W468F MSDH.

Quantification of the free cysteine content using 2PDS (2,2'-dipyridyl disulphide)

Free cysteine content in the wild-type and QCys MSDHs were deduced from the absorbance of pyridine-2-thione using an absorption coefficient of $8080\text{ M}^{-1}\cdot\text{cm}^{-1}$ at 343 nm [17]. The

enzyme was dissolved in 50 mM potassium phosphate buffer, pH 8.2, and 2PDS was added in excess relative to the enzyme.

Kinetic parameters of wild-type and QCys 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), or 50 mM Tes (pH 8.2). The initial-rate data were fitted to the Michaelis–Menten relationship using 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: 500 μM MMSA or 1 mM MSA, 12 mM NAD and 500 μM CoA.

Kinetic studies of tryptophan fluorescence quenching

The time-course of MSDH fluorescence quenching induced by NAD-binding was recorded on a SAFAS flx spectrofluorimeter maintained at 30 °C after addition of 2 mM NAD to 1.25 μM wild-type and mutant MSDHs in 50 mM potassium phosphate, pH 8.2. Absorption and emission wavelengths were set at 297 and 335 nm respectively. For NAD-dependence of the conformational rearrangement, the k_{obs} (pseudo-first-order) values were determined at each NAD concentration by fitting the slow Trp⁴⁶⁸ fluorescence quenching to a monoexponential expression. Data were then fitted to equation (1) to determine K_{app} and $k_{\text{obs max}}$:

$$k_{\text{obs}} = (k_{\text{obs max}} [\text{NAD}]) / (K_{\text{app}} + [\text{NAD}]) \quad (1)$$

pH-dependence of MSDH reactions with 2PDS and IAM (iodoacetamide)

Due to the high reactivity of Cys³⁰² with 2PDS in the Qcys MSDHs, kinetic measurements were carried out on a SX18MV-R stopped-flow apparatus (Applied PhotoPhysics) at 30 °C, under pseudo-first-order conditions over a pH range 5.5–9 with a mixed 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). One syringe contained MSDH in the presence or absence of 4 mM NAD, and the other contained 2PDS. When Cys³⁰² reactivity was low i.e. in the presence of NAD, rate measurements were carried out using a SAFAS UV mc2 spectrophotometer, under the experimental conditions described above.

Alkylation rates for wild-type and QCys MSDHs using IAM were determined over a pH range of 5.5–9 in buffer A at 30 °C under pseudo-first-order conditions, by following the loss of activity. Inactivation of holo-(wild-type) and QCys MSDHs were carried out in the presence of 2 mM NAD. In all experiments, the pseudo-first-order and second-order rate constants, k_{obs} and k_2 respectively, were determined at each pH, and data were analysed as described by Marchal and Branlant [8].

HPLC analysis of MSDH reaction products in the absence of CoA

Identification of MSDH reaction products in the absence of CoA was carried out using HPLC. Reaction mixtures contained wild-type MSDH (4 μM), MMSA (500 μM), or MSA (400 μM) and NAD (2 mM). Progress curves of NADH production in 50 mM potassium phosphate (pH 8.2) were followed at 30 °C. After completion of the reaction, the enzyme was precipitated by addition of 0.2 M HCl and eliminated by centrifugation. A fraction of the supernatant was then injected into a IOA2000

column (Alltech®) followed by isocratic elution using 0.1 M HCl. Products were monitored by refractometry using a SP 8430 RI detector (Spectra-Physics®) and MMSA, MSA, propionic acid, acetic acid, methylmalonic acid and malonic acid were used as standards.

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 analysed using the SX18MV-R software package.

Analysis of the overall steps leading to thioacylenzyme formation

Progress curves of NADH production were recorded in 50 mM potassium phosphate (pH 8.2), in the presence or absence of CoA at 30 °C. One syringe was filled with MSDH (4 μM) and NAD (4 mM) and the other contained MMSA at various concentrations. Data were fitted to equation (2) to determine k_{ac} and K_{s} values, where S represents the substrates MMSA or MSA.

$$k_{\text{ac}} = (k_{\text{ac max}} [\text{S}]) / (K_{\text{s}} + [\text{S}]) \quad (2)$$

For pH-dependence studies, data were collected at 10 or 30 °C in buffer A and analysed as described by Marchal et al. [10].

NADH-dissociation rate from the thioacylenzyme–NADH complex

Experiments were carried out in the absence of CoA in 50 mM potassium phosphate (pH 8.2) at 30 °C. 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 MSDH (7.5 or 15 μM) and NAD (4 mM), and the other contained either MMSA (1 mM) or MSA (2 mM), LDH (7.5 μM) and pyruvate (20 mM).

Decarboxylation rate of the thioacylenzyme intermediate

The rate of the decarboxylation process was evaluated at 30 °C by using the PEP/PEPC/MDH system as a coupled assay. To minimize the background level of carbon dioxide present, nitrogen was bubbled through the stock potassium phosphate, MgCl_2 and PEP solutions. The reaction mixture containing 50 mM Tes (pH 8.2), 10 mM MgCl_2 , 0.4 mM NADH, 4 mM PEP, 100 units/ml MDH and 10 units/ml PEPC was divided into two equal volumes. MSDH (3 or 6 μM , final concentrations) and NAD (2 mM) were added to one volume, and MMSA (1 mM) to the second. The two volumes were pre-incubated separately for 5 min to remove residual bicarbonate, and then mixed together. NAD reduction and NADH oxidation were recorded at 340 nm. The total rate of formation of both bicarbonate and carbon dioxide was assayed by supplementing the reaction mixture with 500 units of bovine erythrocyte carbonic anhydrase/ml.

RESULTS

Evidence for a lag-phase

Progress curves for enzymatic turnover exhibited an initial lag-phase, which prevented detailed kinetic and mechanistic characterization of the catalytic pathway. As shown in Figure 1, the steady-state rate was attained after approx. 5 min when the reaction was initiated by addition of apo-(wild-type) MSDH. The lag-phase was eliminated after pre-incubation of the apoenzyme with NAD. By contrast, pre-incubating the apo-form with MMSA

Figure 1 Progress curves for enzymatic turnover of wild-type MSDH from *B. subtilis*

Assays were performed at 30 °C under the following conditions: 50 mM potassium phosphate buffer (pH 8.2), 2 mM NAD, 500 μ M MMSA and 500 μ M CoA. In curve A, the reaction was initiated by adding 0.125 μ M apo-MSDH and the steady-state rate was attained after approx. 5 min (solid line). In curve B, the enzyme pre-incubated for 20 min in the presence of 2 mM NAD was added to the complete assay to initiate the reaction.

or CoA at concentrations much higher than the K_m values had no effect. Moreover, pre-incubating apo-MSDH with both NAD and MMSA did not diminish the duration of the lag-phase when determined with NAD alone. Therefore MSDH activation appears to be promoted only by NAD. The simplest model capable of explaining the enzyme activation is:

where E and E' are the inactive and active forms of the enzyme respectively. To gain more insight into the role of NAD in the enzyme activation process, the quenching of tryptophan fluorescence intensity upon cofactor binding was evaluated. When apo-MSDH was excited at 297 nm, a fluorescence emission maximum was observed at 335 nm (results not shown). Addition of NAD led to fluorescence quenching, which is in fact the consequence of two additive effects: (i) the formation of the binary E $\cdot$ NAD complex upon NAD binding to the apo-structure, which results in 75 % of the quenching and the rate of which cannot be attained even when using a stopped-flow apparatus, and (ii) a slow phase which is time-dependent and represents 25 % of the quenching (Figure 2). This experiment was carried out using several NAD concentrations ranging from 0–5 mM, and at each NAD concentration a k_{obs} value was calculated by fitting the slow additional fluorescence quenching to a mono-exponential equation. This allowed the determination of a K_{app} of 0.38 mM for NAD and a $k_{\text{obs max}}$ of 0.18 min^{-1} (Figure 3). The $k_{\text{obs max}}$ value is consistent with the duration of the lag-phase when the MSDH apo-form is used for activity assays (Figure 1). Therefore the slow phase is likely to be representative of enzyme activation. In this context, the NAD concentration-dependence of this phase is in agreement with mechanism I.

MSDH from *B. subtilis* contains five tryptophan residues. Analysis of the fluorescence properties of each of the W28F, W76F, W177F, W397F, and W468F MSDH mutants revealed that time-dependent additional quenching is due only to Trp⁴⁶⁸ (Figure 2).

Kinetics of the reaction of apo- and holo-(wild-type) and QCys MSDHs with 2PDS and IAM

The reaction of 2PDS with apo-(wild-type) MSDH led to the formation of 4 moles of pyridine-2-thione per monomer under

Figure 2 Time-course of the MSDH fluorescence change induced by NAD-binding

Fluorescence quenching was recorded after addition of 2 mM NAD to 1.25 μ M wild-type (WT) and W468F MSDHs dissolved in 50 mM potassium phosphate buffer, pH 8.2 at 30 °C. The inset details the fluorescence quenching recorded for the W28F, W76F, W177F and W397F MSDHs under the same experimental conditions. Excitation and emission wavelengths were 297 and 335 nm respectively. The raw fluorescence quenching data were converted into relative fluorescence intensities using the fluorescence intensity of the apo-forms as a reference.

Figure 3 Effect of NAD concentration on the rate of the apo $\rightarrow$ holo transition

Apo-(wild-type) MSDH (1.25 μ M) was incubated in the presence of NAD (0.25–5 mM) in 50 mM potassium phosphate buffer, pH 8.2 at 30 °C. The time-course of the MSDH fluorescence change induced by NAD-binding was then recorded at 335 nm. At each NAD concentration a k_{obs} value was calculated by fitting the slow additional fluorescence quenching to a mono-exponential equation. Data were fitted to eqn (1) demonstrating a K_{app} of 0.38 ± 0.08 mM and a $k_{\text{obs max}}$ of 0.18 ± 0.01 min^{-1} .

native conditions, as determined from the absorbance change at 343 nm. This strongly suggested that, in addition to the catalytic Cys³⁰² that is expected to be reactive, three other cysteine residues in the apo-structure were also reactive. MSDH from *B. subtilis* contains six cysteines at positions 49, 176, 302, 305, 369 and 430. Therefore to selectively study the chemical reactivity of Cys³⁰² towards 2PDS, we substituted alanine for all the cysteine residues, except Cys³⁰². The fact that QCys MSDHs had catalytic parameters similar to those of the wild-type enzyme (see below) suggested that the substitutions did not cause significant effects at either the structural or enzymatic levels. Thus QCys MSDHs could be considered as representative of the wild-type enzyme, in order to study the pH-dependence of the second-order rate constant k_2 for the reaction of Cys³⁰² with 2PDS. As shown in Figure 3 and Table 1, the $\text{p}K_{\text{app}}$ of approx. 8.7 obtained for the

Table 1 Parameters for the pH-dependence of the second-order rate constants for the reaction of Cys³⁰² with 2PDS and IAM for the wild-type and QCys MSDHs

Kinetics of the reaction of Cys³⁰² with 2PDS and IAM were determined over a pH range of 5.5–9 at 30 °C. The experimental conditions are described in the Experimental section. The second-order rate constants at each pH were fitted to a monosigmoidal equation using Sigmaplot (SPSS) to determine the pK_{app} s. The error estimates are those provided by the program.

Enzyme	Cys ³⁰² k_2 ($M^{-1} \cdot s^{-1}$)	pK_{app}
Apo-MSDHs		
Wild-type		
+ IAM	30 ± 8	8.95 ± 0.15
QCys		
+ IAM	16 ± 5	8.80 ± 0.20
+ 2PDS*	$(1.4 \pm 0.1) \times 10^5$	8.70 ± 0.05
	$(1.8 \pm 0.1) \times 10^4$	8.75 ± 0.05
Activated-MSDHs†		
Wild-type		
+ IAM	19 ± 1	8.15 ± 0.05
QCys		
+ IAM	9 ± 1	8.10 ± 0.10
+ 2PDS*	$(5.1 \pm 0.3) \times 10^4$	8.60 ± 0.10
	77 ± 2	7.90 ± 0.10

* Kinetic transients showed the contribution of two populations of Cys³⁰² present in equal concentration within the tetramer with two cysteines exhibiting a chemical reactivity higher than the other two.

† Activated forms refer to wild-type and QCys MSDHs pre-incubated for 20 min with 2 mM NAD at 30 °C in 50 mM potassium phosphate buffer, pH 8.2.

apo-QCys MSDH represents the contribution of two populations of Cys³⁰² that are present in equal concentrations within the tetramer, with k_2 values of $1.4 \times 10^5 M^{-1} \cdot s^{-1}$ for two cysteines, and $1.8 \times 10^4 M^{-1} \cdot s^{-1}$ for the other two respectively. A similar behaviour was observed for the holo-form. Indeed, two cysteine residues exhibited a k_2 of $77 M^{-1} \cdot s^{-1}$, which is at least 2 orders of magnitude lower when compared with the apo-form, whereas the two remaining cysteine residues were still reactive, with a k_2 value of $5.1 \times 10^4 M^{-1} \cdot s^{-1}$ (Figure 4 and Table 1). Moreover, the pK_{app} of the less reactive cysteine residues shifted to 7.9 whereas the pK_{app} of the other two was not significantly changed.

Another means by which to characterize Cys³⁰² reactivity was to follow the loss of activity upon IAM alkylation. In this case, only the Cys³⁰² belonging to the 'active' subunits is probed. The inactivation process of the apo-(wild-type) and QCys MSDHs obeyed pseudo-first-order kinetics. The pH-rate profiles showed a sigmoidal behaviour with a pK_{app} of 8.95 and 8.80, and k_2 values of 30 and $16 M^{-1} \cdot s^{-1}$ for apo-(wild-type) and QCys MSDHs respectively (Table 1). For both holo-forms, Cys³⁰² exhibited a pK_{app} of approx. 8.1, close to the value (7.9) obtained for the two less reactive Cys³⁰² residues in QCys MSDHs when using 2PDS. The k_2 values for holo-forms were approx. 2-fold lower than those of the apo-forms (Table 1).

Steady-state kinetic parameters of wild-type and QCys MSDHs

Prior to any kinetic studies, wild-type or QCys MSDHs were activated in the presence of NAD. The kinetic parameters determined at the optimum pH of 8.2 and at 30 °C for the MSDH-catalysed reaction under steady-state conditions are summarized in Table 2. The wild-type enzyme displayed K_m values of 2.3 mM and 1.78 mM for NAD, and 0.1 mM and 0.03 mM for CoA using MMSA and MSA as substrates respectively, and the k_{cat} value was 6.7-fold higher when using MSA as the substrate. The kinetic parameters for the QCys MSDHs are similar to those of the wild-type enzyme. The K_m values for NAD and MMSA were 1.8-fold lower and 2.1-fold higher respectively, whereas the K_m

Figure 4 pH-dependence of the second-order-rate constant k_2 for the reaction of Cys³⁰² with 2PDS on QCys MSDH

Kinetic studies were performed over a pH range of 5.5–9.0 or 6.0–9.0 in buffer A at 30 °C. The MSDH concentration was 1.55 μM and 2PDS was 20- or 30-fold in excess relative to the free-SH concentration. Experimental data were analysed as described by Marchal and Branlant [8]. (A) Transients obtained on the apo-form were best described by a double exponential expression reflecting the contribution of two populations of non-equivalent Cys³⁰² present in equal concentrations (at pH ≤ 6.5 , only one population was observed). Both data sets were fitted against a one-pKa model, which gave a pK_{app} of approx. 8.7 but different k_2 values of $1.4 \times 10^5 M^{-1} \cdot s^{-1}$ (●) and $1.8 \times 10^4 M^{-1} \cdot s^{-1}$ (◆) respectively. (B) As described for the apo-form, two populations of Cys³⁰² were also characterized in the holo-form. Data sets were fitted against a one-pKa model, which gave k_2 and pK_{app} values of $77 M^{-1} \cdot s^{-1}$ and 7.90 (■), and $5.1 \times 10^4 M^{-1} \cdot s^{-1}$ and 8.60 (▲) respectively. As described above, the two Cys³⁰² populations were not distinguishable at pH ≤ 6.5 . Thus the k_2 value of approx. $20 M^{-1} \cdot s^{-1}$ exhibited by the 'activated' Cys³⁰² (■) at pH ≤ 6.5 reflects the contribution of the 'non-activated' population (▲) (k_2 approx. $130 M^{-1} \cdot s^{-1}$ at pH 6.0). Therefore the pK_{app} value of 7.9 is slightly overestimated.

for CoA and the k_{cat} remained unchanged. In the absence of CoA, the steady-state rate constant (k_{H_2O}) of $10^{-3} s^{-1}$ reflects the poor efficiency of the hydrolytic process in the MSDH-catalysed reaction compared with the transthioesterification step.

Determination of the rate-limiting step

Prior to any single turnover experiments, wild-type or QCys MSDHs were activated in the presence of NAD.

Analysis of the overall step leading to thioacylenzyme formation

Under pre-steady-state conditions, at pH 8.2 and 30 °C, fast kinetic experiments showed a burst of NADH production at a concentration of 2 mM NAD and sub-saturating MMSA or MSA concentrations. A similar k_{obs} of approx. $500 s^{-1}$ was measured for the wild-type and QCys MSDHs in the presence or absence of CoA irrespective of the substrate used. These results indicate that the rate-limiting step takes place after hydride transfer and show that the acylation process is CoA-independent. The burst

Table 2 Kinetic parameters of the wild-type and QCys MSDH-catalysed reactions under steady-state conditions

Kinetic parameters were deduced from non-linear regression of experimental data sets, according to the Michaelis–Menten model; k_{cat} values are expressed per subunit (considering four active subunits per tetramer). The steady-state initial rates of the reaction of wild-type and QCys MSDHs were measured at 30 °C in 50 mM potassium phosphate buffer, pH 8.2. As mentioned in the Experimental section, the determination of the decarboxylation rate was performed in 50 mM Tes, pH 8.2. Thus control experiments were performed in the same buffer and, as expected, the kinetic parameters obtained were similar to those obtained using potassium phosphate buffer. $k_{\text{H}_2\text{O}}$ values represent the steady-state rate constants of the reaction in the absence of CoA.

MSDH	K_m MMSA (mM)	K_m NAD (mM)	K_m CoA (mM)	k_{cat} (s^{-1})	$k_{\text{H}_2\text{O}}$ (s^{-1})
Wild-type	0.06 ± 0.01	2.30 ± 0.06	0.12 ± 0.02	1.1 ± 0.1	10^{-3}
QCys	0.17 ± 0.03	1.22 ± 0.14	0.10 ± 0.03	1.25 ± 0.12	10^{-3}
	K_m MSA (mM)	K_m NAD (mM)	K_m CoA (mM)	k_{cat} (s^{-1})	$k_{\text{H}_2\text{O}}$ (s^{-1})
Wild-type	0.12 ± 0.01	1.78 ± 0.39	0.030 ± 0.01	7.4 ± 0.3	10^{-3}

Figure 5 Burst kinetics for the MSDH-catalysed reaction

The experiment was carried out under pre-steady-state conditions in 50 mM potassium phosphate buffer (pH 8.2) at 30 °C and in the absence of CoA. Under these experimental conditions, accumulation of the thioacylenzyme is observed due to the very low steady-state rate, $10^{-3} \cdot \text{s}^{-1}$. After mixing, the reaction contained 2 mM NAD, 500 μM MMSA and 4 μM (curve A) or 8 μM (curve B) of 'activated'-MSDH. The burst of NADH production was monitored at 340 nm. The extrapolated intercept from the steady-state rate portion corresponds to a burst of 0.5 mol of NADH/mol of MSDH monomer.

magnitude observed during the first turnover with both substrates showed that only 2 mol of NADH were formed per mol of tetramer (Figure 5). As a control experiment, NAD concentration was increased to 20 mM, but this did not modify the magnitude of NADH production and the k_{obs} . This burst magnitude is related to both the number of functional active sites and the $k_{\text{ac}}/k_{\text{cat}}$ or $k_{\text{ac}}/k_{\text{H}_2\text{O}}$ ratios. Considering the values given in Tables 2 and 3, the theoretical burst magnitude should be 4 for tetrameric MSDH. Therefore the data clearly indicate that wild-type and QCys MSDHs exhibited half-of-the-sites reactivity.

The pH-dependence of the acylation rate constant for the wild-type enzyme was determined in the absence of CoA. To avoid any k_{ac}/K_s contribution to the pH– k_{ac} curve, the experiment should be carried out at a saturating MMSA concentration over the full pH range (5.0–8.5). Owing to the fact that the acylation rate appeared to be too fast at 30 °C, the experiments were performed at 10 °C. The apparently similar K_s value of 3.6 mM determined at both pH 5.0 and 8.5, which is 60-fold higher than the K_m obtained under steady-state conditions (Table 3), suggested no variation in the K_s value within the pH range 5.0–8.5. At an MMSA concentration of 10 mM which was found to be saturating, the pH– k_{obs} profile exhibited a sigmoidal curve that was related to the contribution of one ionizable group of $\text{p}K_{\text{app}}$ 5.4 that must be deprotonated for acylation, and characterized by a k_{ac} of 450 s^{-1} (Figure 6, see legend). The same experiment was also performed at 30 °C and at sub-saturating substrate concentrations i.e. 0.5 mM MMSA and 0.4 mM MSA. Under these conditions, the pH– k_{ac} profile

Table 3 Parameters for the pH-dependence of wild-type and QCys MSDHs pre-steady-state kinetics

$k_{\text{ac max}}$ and K_s values were deduced from non-linear regression analysis of experimental data collected at 10 or 30 °C on a SX18MV-R stopped-flow apparatus, according to equation (2). Other experimental data are given in the Experimental section. pH-dependence experiments were carried out in buffer A at 10 and 30 °C (for more details see the Experimental section). Data were analysed by non-linear regression analysis against a one- $\text{p}K_a$ model.

MSDH	$k_{\text{ac max}}$ (s^{-1})	K_s (mM)	$\text{p}K_{\text{app}}$ Cys ³⁰²
Wild-type			
MMSA			
10 °C	450 ± 10	3.6 ± 0.2	$5.4 \pm 0.1^*$
30 °C	$1200 \pm 180\ddagger$	$3.5 \pm 1.0\ddagger$	$5.7 \pm 0.1\ddagger$
MSA			
30 °C	$2900 \pm 900\ddagger$	$4.7 \pm 1.8\ddagger$	$6.0 \pm 0.1\ddagger$
QCys			
MMSA			
30 °C	480 ± 20	2.2 ± 0.2	$5.9 \pm 0.1\ddagger$

* Experiment carried out at saturating concentrations of MMSA.

† Experiments performed using sub-saturating concentrations of MMSA (0.5 mM) or MSA (0.4 mM).

‡ At greater than 3 mM MMSA or 2 mM MSA, acylation appears to be too fast at 30 °C to allow accurate determination of k_{ac} values. The $k_{\text{ac max}}$ and K_s values were then estimated by extrapolation to saturating MMSA or MSA concentrations and fitting experimental data to eqn (2).

Figure 6 Representative pH-dependence of the acylation rate constant k_{ac} for the wild-type MSDH-catalysed reaction

Pre-steady-state data were collected at 10 °C on a SX18MV-R stopped-flow apparatus (Applied PhotoPhysics) using the 5 μl cell, by rapidly mixing 4 μM wild-type MSDH, 2 mM NAD and 10 mM MMSA (final concentrations) over a pH-range of 5–8.5 in buffer A. At pHs lower than 5, MSDH was not stable. NADH appearance was monitored at 340 nm and experimental data (●) were best fitted by non-linear regression analysis against a one- $\text{p}K_a$ model, identified by the best-fit theoretical curve (solid line).

Figure 7 Representative transient for the determination of (A) the NADH dissociation rate and (B) the decarboxylation rate from the wild-type thioacylenzyme intermediate with MMSA as substrate

(A) A solution of $7.5 \mu\text{M}$ LDH, 20 mM pyruvate and 1 mM MMSA was rapidly mixed with an equal volume of $7.5 \mu\text{M}$ 'activated'-MSDH and 4 mM NAD. Both syringes contained 50 mM potassium phosphate buffer, pH 8.2. Under the same experimental conditions, the rate of oxidation of free NADH by LDH was shown to be 130 s^{-1} . Data collected were best fitted to a triphasic expression using the Origin 7 software (Microcal), as shown by the lack of systematic deviation in the plot of residuals (lower panel). The first phase represents the acylation step, the second one the NADH consumption after its release from the thioacylenzyme–NADH complex and the third one could be due to the reverse LDH-catalysed reaction. The rate constants obtained for the global fitting are $72 \pm 1 \text{ s}^{-1}$, $56 \pm 1 \text{ s}^{-1}$, and $(3.00 \pm 0.07) \times 10^{-2} \text{ s}^{-1}$ respectively. (B) The rate of the decarboxylation process was determined at 30°C from the thioacylenzyme intermediate by using the PEP/PEPC/MDH system as a coupled assay. Experimental conditions are described in detail in the Experimental section. Data collected using a split time-base (2000 points for the first 100 ms, and 2000 points for the remaining 10 s) were best fitted to a triphasic expression using the Origin 7 software as shown by the lack of systematic deviation in the plot of residuals (lower panel). The first phase represents the acylation step, the second one the bicarbonate release and the third one could be due to the reverse MDH-catalysed reaction. The rate constants obtained for the global fitting are $415 \pm 5 \text{ s}^{-1}$, $2.5 \pm 0.1 \text{ s}^{-1}$, and $(1.00 \pm 0.01) \times 10^{-3} \text{ s}^{-1}$ respectively.

could also include a $k_{\text{ac}}/K_{\text{S}}$ contribution. In fact, similar $\text{p}K_{\text{app}}$ values were obtained (5.7 and 6.0) using MMSA and MSA respectively. As expected, data obtained from QCys MSDHs and a sub-saturating concentration of MMSA at 30°C did not differ significantly from those of the wild-type MSDH, with similar $\text{p}K_{\text{app}}$ and $k_{\text{ac max}}$, and K_{S} values that were decreased only 2.5-fold and 1.6-fold respectively (see comment, Table 3).

NADH release from the thioacylenzyme–NADH complex is not rate-limiting

The rate of NADH dissociation from the thioacylenzyme–NADH complex was determined using LDH as an NADH-trapping system. The experiment was performed using wild-type MSDH at pH 8.2 and 30°C under pre-steady-state conditions and in the absence of CoA. The resulting progress curves were fitted to a triphasic expression: the first kinetic phase represents the burst of NADH production associated with the acylation process, whereas the second corresponds to the titration of the NADH that dissociates from the thioacylenzyme–NADH complex, and the third in which the rate is very low, could be due to the reverse LDH-catalysed reaction. Under the experimental conditions used (see Figure 7A), the rate of NADH oxidation by LDH (130 s^{-1}) was higher than the apparent rate constants of 56 s^{-1} (Figure 7A) and 50 s^{-1} (results not shown) determined for the second kinetic phase using MMSA and MSA as substrates respectively. Thus these apparent rate constants can be assigned to the NADH release from the thioacylenzyme–NADH complex. These rates – which are likely to be underestimated values of the intrinsic rates of NADH release – are 51-fold and 7-fold higher than the k_{cat} values using MMSA and MSA as substrates respectively.

Determination of the decarboxylation rate and identification of bicarbonate as a product of the MSDH-catalysed oxidation of MMSA

In the absence of CoA, HPLC analysis identified propionate and acetate as the products of the wild-type-catalysed reactions with MMSA and MSA as substrates respectively. This result suggested that the decarboxylation process precedes CoA-binding. To validate this assumption, a coupled assay was used to determine (i) the rate of the decarboxylation of the thioacylenzyme intermediate and (ii) whether carbon dioxide or bicarbonate is released as a final product. For this assay, PEPC was used as a bicarbonate (but not carbon dioxide)-trapping system to convert PEP into oxaloacetate, the reduction of which into malate by MDH was monitored by NADH disappearance at 340 nm (see Figure 7B). Again, this experiment was performed at pH 8.2 and 30°C under pre-steady-state conditions and in the absence of CoA. A rate of 2.5 s^{-1} was found for the rate of bicarbonate formation. This value is much higher than the rate of noncatalysed hydration of carbon dioxide, which is approx. 0.04 s^{-1} at room temperature [18]. Therefore this demonstrates that bicarbonate is the end product of thioacylenzyme decarboxylation. Moreover, the rate of bicarbonate formation is similar to the k_{cat} values of wild-type and QCys MSDH-catalysed reactions (Table 2). Indeed, as described in Table 2, k_{cat} values were expressed per subunit taking into account four active subunits per tetramer. If we consider the half-of-the-sites reactivity, the 'true' k_{cat} value is 2.2 s^{-1} .

DISCUSSION

MSDH from *B. subtilis* exhibits a marked lag-phase in activity assays. Binding of NAD to the apo-form promotes an activation step that converts the enzyme from an inactive form to an active one. Thus a conformational rearrangement is likely to accompany the activation process. This is supported by a modification of the Trp⁴⁶⁸ environment as shown by the additional time-dependent fluorescence quenching observed upon NAD binding. Inspection of known three-dimensional structures of non-phosphorylating CoA-independent ALDHs [1–3] shows that position 468 is located at the dimer interface. Therefore the change in the Trp⁴⁶⁸ fluorescence properties in MSDH likely reflects a local rearrangement within the dimer interface. However, the molecular and

structural factors which are responsible for MSDH activation remain to be characterized. In this context, a coenzyme-induced disorder-to-order transition of the loop containing residue 468, similar to that reported by Hurley et al. [19] on the human mitochondrial ALDH2, is a possibility. The observation that after one turnover the lag is suppressed suggests that MSDH undergoes structural imprinting. *In vitro*, this imprinting appears to be promoted only by NAD. No effect is observed in the presence of MMSA that would support a kinetic mechanism whereby NAD acts as the first-binding substrate followed by MMSA (or MSA) as the second substrate. The lag-phase observed with MSDH has already been reported for yeast ALDH, for which the rate of the conformational rearrangement induced by NADP-binding was shown to be accelerated in the presence of Mg^{2+} [20,21]. However, this is not a general feature since the MSDH-activation process does not exhibit such a dependence towards divalent cations such as Mg^{2+} or Ca^{2+} (results not shown). The question regarding the physiological significance of the lag-phase in MSDH still remains to be addressed.

MSDH from *B. subtilis* exhibits a half-of-the-sites reactivity. That is supported by the stoichiometry of NADH formation of 2 moles per tetramer in the absence of CoA. Since the presence of CoA had no effect on the rate of the acylation step, the value of 2 moles of NADH per tetramer is representative of what occurs in the presence of CoA. The half-of-the-sites reactivity shows that only two active sites per tetramer are functional and therefore raises the question as to whether two populations of active sites already pre-exist in the apo- and holo-enzymes. This seems to be the case at least for the catalytic Cys³⁰². First, kinetic data obtained for the apo-QCys MSDH, using 2PDS as a specific cysteine probe, reveal the contribution of two Cys³⁰² species exhibiting a pK_{app} of approx. 8.7, but different reactivities. Second, formation of the holo-form induces an at least 230-fold decrease in the reactivity of two Cys³⁰² residues per tetramer with a pK_{app} shift from approx. 8.7 to 7.9, whereas it has little effect on the reactivity and the pK_{app} of the two remaining Cys³⁰² residues. The IAM inactivation data, from which a pK_{app} of 8.1 was determined, provide strong evidence for the assignment of the 7.9 pK_{app} to the Cys³⁰² belonging to the two 'active' subunits.

Among the non-phosphorylating ALDH family members, there are few that share this property of the half-of-the-sites reactivity. It has been considered as an extreme example of negative cooperativity [19,22], but the structural basis for this cooperativity is still not understood. Studies of the tetrameric human liver mitochondrial ALDH have led to the proposal of a model where there is one functioning and one non-functioning subunit in each dimer pair [22]. It is tempting to apply such a model to the tetrameric MSDH from *B. subtilis*.

The k_{ac} -versus-pH curve fits to a monosigmoidal profile with a pK_{app} between 5.4 and 6.0 depending on the substrate used. To be active, Cys³⁰² of MSDH should exist in the deprotonated form. Since the transient hemithioacetal intermediate is not protonated, due to the presence of an oxyanion hole, the pK_{app} value of 5.4–6.0 can be assigned to Cys³⁰². It is also expected that in the pH range 5–8.5, the carboxyl group of the substrate does not contribute to the k_{ac} -versus-pH curve as the protein environment composed of Arg¹²⁴ and Arg³⁰¹ (C. Stines-Chaumeil, unpublished work) is likely to decrease its pK_{app} to a value of less than 4.5. The pK_{app} of 5.4–5.7 for Cys³⁰² in the ternary NAD–MMSA–MSDH complex strongly suggests that formation of the ternary complex induces an additional local conformational rearrangement within the active site, leading to a decrease of the Cys³⁰² pK_{app} value by more than 2 units, when compared with the MSDH–NAD binary complex. Thus the Cys³⁰² activation mode appears to be sequential in MSDH from *B. subtilis* with a Cys³⁰² pK_{app} shift from approx. 8.8 in the

apo-form to approx. 8.0 in the binary complex and finally 5.4–5.7 in the ternary complex. A notable feature of this activation process is the low reactivity of the two catalytic Cys³⁰² residues in the holo-form. However, this does not prevent the acylation step from being fast and not rate-limiting.

As shown in the Results section, the acylation process is not rate-limiting because the acylation rate, determined in the presence or absence of CoA, is much higher – at least 350-fold – than the k_{cat} value. Thus the rate-limiting step follows NADH production, although one needs to bear in mind that *in vitro* the 'true' rate-limiting process is associated with the structural imprinting reaction (the kinetic studies can be considered as representative of the *in vivo* situation in *B. subtilis*. Indeed, the NAD concentration is likely to be such that MSDH is in an MSDH–NAD form). The fact that the apparent dissociation rate constants for NADH release from the thioacylenzyme–NADH complex were higher than the k_{cat} values showed that this step is not rate-limiting and occurs prior to transthioesterification. Thus the rate-limiting step is associated with either the β -decarboxylation step, the transthioesterification, the release of the product or any of the potential conformational changes. In fact, the β -decarboxylation process is likely to be rate-limiting for the overall reaction. This is supported by the value of 2.5 s⁻¹ for the rate of bicarbonate formation determined with MMSA as a substrate, which is similar to the k_{cat} value (see Table 1). The fact that bicarbonate is the end product of the thioacyl enzyme decarboxylation is confirmed by the values of the decarboxylation rate which are similar in the presence or absence of carbonic anhydrase. The question which now arises is related to the MSDH-catalysed formation of the bicarbonate. A possible mechanism would result in the direct release of bicarbonate product as proposed for the β -ketoacyl synthase domain of a multifunctional fatty acid synthase [23]. An alternative mechanism would require the inclusion of an additional step for hydration of carbon dioxide after decarboxylation. Finally, our studies also show that the decarboxylation step occurs on the thioacylenzyme intermediate, and thus leads to formation of a new thioacylenzyme species that should be competent to undergo a nucleophilic attack by a CoA molecule to form the activated-CoA ester product. The proposed ping-pong mechanism (Scheme 2) is in accordance with the kinetic mechanism described for the acetaldehyde and the succinate dehydrogenases, which also belong to the CoA-dependent ALDH family [24,25].

As indicated in the Introduction section, the MSDH from *B. subtilis* belongs to the structural family of the non-phosphorylating ALDHs. However, as shown in the present study, MSDH from *B. subtilis* exhibits several peculiar properties when compared with GAPN from *Streptococcus mutans*, a CoA-independent ALDH extensively studied by our laboratory. First, the lag-phase exhibited during the enzymatic turnover in MSDH is absent in GAPN. Second, the Cys³⁰² activation mode is different. As described above, the ternary-complex formation is essential for full Cys³⁰² activation in MSDH. In GAPN, the scenario is clearly different. Formation of the NADP–GAPN binary complex is sufficient to achieve full activation of the catalytic Cys³⁰², although addition of the substrate kinetically favours formation of a competent ternary complex [8]. Thus it appears that among the non-phosphorylating ALDH family, evolution has led to different solutions to achieve the chemical activation of the catalytic Cys³⁰². Third, both tetrameric enzymes are composed of two populations of non-equivalent active sites, but in GAPN all the subunits are functional although they differ in catalytic efficiency (A. Pailot, personal communication). Finally, MSDH exhibits two other major differences: its active site is adapted to catalyse a β -decarboxylation of the substrate and a transthioesterification

process. This explains why the ordered kinetic mechanism of GAPN, in which NADPH dissociates last, is not relevant to the MSDH mechanism in which NADH release precedes the transthiioesterification. In this context, it would be informative to know how the CoA molecule is recognized by MSDH and in particular whether and how the NADH- and CoA-binding sites overlap with each other. Determination of the three-dimensional structures of MSDH from *B. subtilis* in complex with its different co-substrates will be very informative.

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