Adenine Binding Mode Is a Key Factor in Triggering the Early Release of NADH in Coenzyme A-dependent Methylmalonate Semialdehyde Dehydrogenase*†

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Background: Conformational dynamics of the cofactor are essential for catalysis by hydrolytic ALDHs.

Results: Crystallographic and kinetic data reveal the molecular basis for NADH release in MSDH, a CoA-dependent ALDH.

Conclusion: Weaker stabilization of the adenine ring triggers early NADH release in MSDH-catalyzed reaction.

Significance: First description of the mechanism whereby the cofactor binding mode is partly responsible for the kinetic behavior of CoA-dependent ALDHs.

Structural dynamics associated with cofactor binding have been shown to play key roles in the catalytic mechanism of hydrolytic NAD(P)-dependent aldehyde dehydrogenases (ALDH). By contrast, no information is available for their CoA-dependent counterparts. We present here the first crystal structure of a CoA-dependent ALDH. The structure of the methylmalonate semialdehyde dehydrogenase (MSDH) from Bacillus subtilis in binary complex with NADH* shows that, in contrast to what is observed for hydrolytic ALDHs, the nicotinamide ring is well defined in the electron density due to direct and H2O-mediated hydrogen bonds with the carboxamide. The structure also reveals that a conformational isomerization of the NNNH is possible in MSDH, as shown for hydrolytic ALDHs. Finally, the adenine ring is substantially more solvent-exposed, a result that could be explained by the presence of a Val residue at position 229 in helix α2, which reduces the depth of the binding pocket and the absence of Gly-225 at the N-terminal end of helix αF. Substitution of glycine for Val-229 and/or insertion of a glycine residue at position 225 resulted in a significant decrease of the rate constant associated with the dissociation of NADH from the MSDH/thioacal Enlighten complex, thus demonstrating that the weaker stabilization of the adenine ring is a key factor in triggering the early NADH release in the MSDH-catalyzed reaction. This study provides for the first time structural insights into the mechanism whereby the cofactor binding mode is responsible at least in part for the different kinetic behaviors of the hydrolytic and CoA-dependent ALDHs.

Among the many enzymes that metabolize aldehydes, the members of the aldehyde dehydrogenase (ALDH) superfamily play a critical role in protecting cells against the cytotoxic and carcinogenic effects of aldehydic compounds. In humans, the clinical importance of ALDHs is supported by the fact that mutations and polymorphisms in ALDH genes lead to defective aldehyde metabolism, the molecular basis of severe diseases (1, 2). Moreover, several ALDHs appear to be markers for normal and cancer stem cells (3, 4). ALDHs are also known to play central roles in many essential biological functions such as intermediary metabolism, embryogenesis, development, and neurotransmission.

The ALDHs catalyze 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 involves the formation of a hemithioacetal intermediate via the nucleophilic attack of the aldehyde function followed by hydride transfer that leads to formation of a thioacal intermediate and NAD(P)H. This intermediate then undergoes a nucleophilic attack by an activated water or CoA molecule. Over the past 15 years both mechanistic and structural aspects of hydrolytic ALDHs have been studied extensively (5–13). In addition to local conformational reorganizations of the active site induced by ligand binding that provide the required flexibility.
Cofactor Dynamics in ALDH Superfamily

for an efficient catalysis (14, 15), one of the key aspects of the chemical mechanism of this ALDH family is the substantial conformational flexibility of the NMN moiety of the cofactor and in particular of the nicotinamide ring. Indeed, the presence of multiple conformations of the NMN moiety is supported by the majority of the x-ray structures of ALDH-NAD(P)⁺ binary complexes as well as NMR studies and has been hypothesized to be due to the peculiar binding mode of the cofactor to a non-canonical Rossmann fold (9, 16–18). Furthermore, significant efforts have been made to characterize the mode by which the cofactor is stabilized during both the acylation and the deacylation steps. In the acylation step, the NMN moiety must be positioned such that an efficient and stereospecific hydride transfer can occur from the hemithioacetal intermediate to the C-4 of the nicotinamide. The side chain of invariant Glu-399 was shown to play an essential role in this stabilization by anchoring the NMN ribose through hydrogen bonds with its hydroxyl groups (19). Moreover, recent studies support a critical contribution of the β-methyl group of the invariant Thr-244 residue that allows the nicotinamide ring to adopt a productive conformation for hydride transfer (20). Nevertheless, this conformation is not suitable for the deacylation because it would sterically preclude the catalytic Glu-268 from playing its role in the hydrolytic process. The fact that the cofactor remains bound to the enzyme along the two-step catalytic mechanism strongly suggested that movement of the reduced NMN (NMNH) moiety of the NAD(P)H is a prerequisite for completion of the second half of the reaction (21). Indeed, the first structural evidence for this conformational change of the NMNH during the catalytic cycle of hydrolytic ALDHs was provided by the crystal structure of a thioacyl-enzyme complex (7). Specifically, this structure revealed that once the acylation step has occurred, the reduced cofactor adopts a new conformation with a flip of the NMNH moiety, which positions the reduced nicotinamide in a conserved cavity that might constitute the exit door for NAD(P)H.

By contrast, less information is available concerning structural and/or mechanistic aspects of the CoA-dependent ALDHs. In an effort to address this gap in knowledge, our group has for several years been studying the catalytic mechanism of the methylmalonate semialdehyde dehydrogenase (MSDH) from Bacillus subtilis (22, 23). This homotetrameric enzyme catalyzes the NAD⁺-dependent oxidation of methylmalonate semialdehyde (MMSA) and malonate semialdehyde to propionate and in particular of the nicotinamide ring.5 Indeed, the presence of multiple conformations of the NMN moiety is supported by the majority of the x-ray structures of ALDH-NAD(P)⁺ binary complexes as well as NMR studies and has been hypothesized to be due to the peculiar binding mode of the cofactor to a non-canonical Rossmann fold (9, 16–18). Furthermore, significant efforts have been made to characterize the mode by which the cofactor is stabilized during both the acylation and the deacylation steps. In the acylation step, the NMN moiety must be positioned such that an efficient and stereospecific hydride transfer can occur from the hemithioacetal intermediate to the C-4 of the nicotinamide. The side chain of invariant Glu-399 was shown to play an essential role in this stabilization by anchoring the NMN ribose through hydrogen bonds with its hydroxyl groups (19). Moreover, recent studies support a critical contribution of the β-methyl group of the invariant Thr-244 residue that allows the nicotinamide ring to adopt a productive conformation for hydride transfer (20). Nevertheless, this conformation is not suitable for the deacylation because it would sterically preclude the catalytic Glu-268 from playing its role in the hydrolytic process. The fact that the cofactor remains bound to the enzyme along the two-step catalytic mechanism strongly suggested that movement of the reduced NMN (NMNH) moiety of the NAD(P)H is a prerequisite for completion of the second half of the reaction (21). Indeed, the first structural evidence for this conformational change of the NMNH during the catalytic cycle of hydrolytic ALDHs was provided by the crystal structure of a thioacyl-enzyme complex (7). Specifically, this structure revealed that once the acylation step has occurred, the reduced cofactor adopts a new conformation with a flip of the NMNH moiety, which positions the reduced nicotinamide in a conserved cavity that might constitute the exit door for NAD(P)H.

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Taken together these data raise important questions regarding the evolution of the catalytic mechanism within the ALDH superfamily. In particular, it is of interest to determine whether the pattern of interactions that is thought to stabilize an efficient hydride transfer conformation of the NMN moiety of the cofactor in the hydrolytic ALDHs is also operative in the members of the CoA-dependent family. Additionally, two key aspects related to the structural dynamics associated with the release of the reduced cofactor remain to be addressed, (i) whether cofactor isomerization after hydride transfer occurs in CoA-dependent ALDHs and (ii) the molecular and structural factors responsible for the early or late release of the reduced cofactor occurring in the catalytic cycle of CoA-dependent and hydrolytic ALDHs, respectively.

We detail here the first crystal structure of a CoA-dependent ALDH. The structure of the binary MSDH/NAD⁺ complex shows that, in contrast to what is observed in nearly all x-ray structures of hydrolytic ALDH-NAD(P)⁺ binary complexes, the nicotinamide ring is well defined in the electron density due to direct and H₂O-mediated hydrogen bonds between the amino group of the carboxamide and residues belonging to the cofactor binding and the catalytic domains. Moreover, the pocket that is postulated to constitute the exit door of the reduced cofactor in the hydrolytic ALDHs is conserved, thus suggesting that a cofactor isomerization also occurs in MSDH and the entire CoA-dependent ALDH family. Finally, superimposition of all ALDH holo-structures reveals that the adenine ring of NAD⁺ is substantially more solvent-exposed in MSDH. Analysis of the adenine binding pocket highlights several structural factors that could explain this marked difference; (i) the presence of a Val residue at position 229 in the MSDH that reduces the depth of the binding pocket, thereby increasing the solvent exposure of the adenine ring, and (ii) the absence of Gly-225 at the N-terminal end of helix α₁. These observations suggest a major role for the adenine binding mode in the dynamics of the reduced cofactor in the MSDH catalytic mechanism. Indeed, substitution of glycine for Val-229 and/or insertion of a glycine residue at position 225 in the MSDH from B. subtilis resulted in a significant decrease of the rate constant associated with the dissociation of NADH from the MSDH/thioacyl-enzyme complex, thus validating this assumption.

EXPERIMENTAL PROCEDURES

Materials—NAD⁺ was purchased from Roche Applied Science. CoA, pyruvate, and lactate dehydrogenase (LDH) were from Sigma. MMSA was synthesized as described by Kupiecki.
and Coon (31), and 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 standard PCR site-directed mutagenesis. Wild-type and mutated MSDHs were produced and purified using a procedure described previously (22). Enzyme concentrations were determined spectrophotometrically by using molar absorption coefficients of 2.04 × 10^5 M\(^{-1}\) cm\(^{-1}\) at 280 nm for wild-type and mutated MSDHs. In the present paper, 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). Before kinetic measurements in the presence of MMSA, wild-type and mutated MSDHs were preincubated at 30 °C with 2 mM NAD\(^+\) to activate the enzyme and eliminate the lag-phase exhibited by progress curves for enzymatic turnover (22). The initial rate data were fit to the Michaelis-Menten equation using nonlinear least-squares regression analysis to determine the Michaelis-Menten equation using nonlinear least-squares enzymatic turnover (22). The initial rate data were fit to the Michaelis-Menten equation using nonlinear 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.

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 Mutated MSDHs—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). Before kinetic measurements in the presence of MMSA, wild-type and mutated MSDHs were preincubated at 30 °C with 2 mM NAD\(^+\) to activate the enzyme and eliminate the lag-phase exhibited by progress curves for enzymatic turnover (22). The initial rate data were fit to the Michaelis-Menten equation using nonlinear 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.

To evaluate the rate of NADH dissociation from the thioacylenzyme-NADH complex, the coupled pyruvate/lactate dehydrogenase (LDH) assay was used as an NADH trapping system. One syringe was filled with 32 \(\mu\)M MSDH and 2 mM NAD\(^+\), and the other contained 2 mM MMSA.

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Crystallization, Data Collection, and Processing—Orthorhombic crystals of the MSDH from B. subtilis were obtained from a concentrated protein solution (200 mg/ml) in the presence of 10 mM NAD\(^+\). Ammonium sulfate was used as precipitant agent. Detailed protocols for MSDH expression, purification, crystallization, and preliminary x-ray analysis were reported previously (32).

Phasing and Refinement—The structure of the MSDH from B. subtilis was solved by molecular replacement, revealing that MSDH crystallizes with one homotetramer per asymmetric unit like the cod liver betaine aldehyde dehydrogenase used as a search model (18) (PDB entry 1bpw). The structure was initially refined using rigid body minimization and simulated annealing procedures before iterative cycles of manual rebuilding and refinement. Using 20–2.5 Å data, the final \(R_{free}\) and \(R_{cryst}\) values were 0.25 and 0.211, respectively. The asymmetric unit consisted of 4 polypeptide chains (in all monomers, residues Glu-3–Phe-486), 4 NAD\(^+\) molecules, and 866 water molecules. The first two residues and the last residue were not found in electron density maps and so are presumably disordered. The MSDH structure exhibited good geometry, with 89.9% of all residues in the most favored and 9.3% in the allowed regions of the Ramachandran plot as indicated by the program Procheck (33). Molecular replacement calculations and structure refinement were carried out using the CNS program package (34). The graphics programs Turbofrodo (35) and PyMOL were used in model building and in preparation of figures, respectively. Further statistics are summarized in Table 1. The refined coordinates of the model of MSDH and the structure factors have been deposited with the Protein Data Bank under accession code 1T90.

### RESULTS AND DISCUSSION

Overall Structure—As expected, the three-dimensional structure of the MSDH from B. subtilis conforms to known tetrameric ALDH structures; the tetramer of the enzyme is a dimer of dimers with strong interactions at the dimer interface but weaker interactions at the tetramer interface (11), and the monomer consists of three domains, the dinucleotide binding domain (residues 3–123 and 141–251), the catalytic domain (residues 252–270), and a small domain (residues 124–140 and 471–486) involved in the oligomerization (Fig. 1). A structural comparison with available entries in the Protein Data Bank database was performed using the PDBeFold server (36). The root mean square deviations upon comparing the \(\text{Ca}\) traces of all available monomer structures vary from 1.4 to 2.5 Å, the most similar and distant structures being that of betaine aldehyde dehydrogenase from *Pseudomonas aeruginosa* (PDB code 2wme (37)) and that of dimeric ALDH3 (PDB code 1ad3 (9)).

### TABLE 1

Statistics of x-ray diffraction data collection and model refinement

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tr>
<td>Space group</td>
<td>P2₁2₁2₁</td>
</tr>
<tr>
<td>Unit-cell parameters (Å)</td>
<td>(a = 195.2), (b = 192.5), (c = 83.5)*</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>20.0-2.5 (2.50-2.59)</td>
</tr>
<tr>
<td>(R_{free}) (%)</td>
<td>13.8 (37.2)*</td>
</tr>
<tr>
<td>((I/\sigma(I)))</td>
<td>9.4 (3.6)*</td>
</tr>
<tr>
<td>Completeness</td>
<td>99.5 (91.5)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>7.3</td>
</tr>
</tbody>
</table>

*From Ref. 32.*
The crystal structure of the MSDH from *B. subtilis* did not reveal significant structural differences between the four monomers within each asymmetric unit. Indeed, the average root mean square deviation value obtained from the different pairwise superimpositions of the C atoms was 0.23 Å, and the monomers exhibited similar average isotropic displacement parameters. Each monomer is occupied by a NAD molecule that is well defined in the final 2Fo−Fc electron density map (supplemental Fig. S1).

**Catalytic Site**—Irrespective of the monomer, the side-chain orientation of Cys-302 is similar to that depicted to be catalytically competent for hydrolytic ALDHs (21). At first sight, this observation contradicts our previous data because NAD binding was shown to elicit a slow conformational change that likely provokes a reorientation of only two Cys-302 residues per tetramer and also a local rearrangement within the dimer interface. Moreover, the presence of two populations of Cys-302 correlated well with the half-site reactivity exhibited by the enzyme (*i.e.* only two subunits are active per tetramer). The apparent discrepancy between solid state and solution data may be explained by either the average crystallographic resolution of the structure or by the much longer time scales for crystallization assays relative to the kinetic experiments. The latter hypothesis is further supported by the low resolution (2.9 Å) x-ray structure of a thioacyl enzyme intermediate recently obtained by soaking the crystals of the binary MSDH/NAD complex with MMSA, which revealed that in each monomer the catalytic Cys-302 exhibits an additional electron density consistent with the presence of a covalently linked substrate molecule (data not shown). Therefore, the quasi-perfect 222 symmetry of the MSDH structure is not helpful in revealing the molecular basis for the half-site reactivity exhibited by the enzyme. In particular, a critical role of a coenzyme-induced disorder-to-order transition of the loop located at the dimer interface, *i.e.* connecting 18 and N (supplemental Fig. S2), as proposed for the human ALDH2, remains to be validated (38).

The nicotinamide ring of NAD is well defined in the electron density maps and is sandwiched between the catalytic Cys-302 on the A-side and the Val-244 and Gly-245 residues on the B-side, respectively. The short distance (3.15 Å) between the sulfur atom of Cys-302 and the C4 atom of the nicotinamide suggests that the NMN portion adopts the so-called “hydride transfer” conformation (21). In addition, the thiol of Cys-302 is positioned between the amide groups of Asn-169 and Asn-427. Asn-169 is invariant within the ALDH superfamily, and its amide group as well as the main-chain nitrogen of Cys-302 were shown to make up an oxyanion hole that allows for an efficient hydride transfer without assistance by a base-catalyst (6). By
contrast, Asn-427 is MSDH-specific and is replaced by Leu in the hydrolytic family (see below and supplemental Fig. S2).

The substrate access tunnel of MSDH can be divided in two parts: a narrow region surrounding the catalytic Cys-302 and a wide region near the tetramer interface. The narrow region can be described as a hydrophobic crown comprising the side chains of Phe-170, Met-173, Trp-177, Met-303, Ala-459, Phe-465, and the aliphatic portion of Arg-301. These seven residues are strictly conserved in the MSDH family except for Ala-459, which is located in the long loop referred to as the substrate specificity loop by Moore et al. (39). Nevertheless, the hydrophobic character of the position 459 is conserved as only Ala and Val are found in known MSDH sequences. The entrance of the narrow part of the catalytic tunnel comprises, notably, the side chains of Arg-124 and Arg-301. Very recently, both residues were shown to participate not only in MMSA binding through stabilizing electrostatic interactions with the carboxylate group of the substrate but likely also in positioning MMSA efficiently relative to Cys-302 in the MSDH/NAD+/MMSA ternary complex (23).

NMN Binding Site—The four NAD⁺ binding sites are occupied by a cofactor molecule and appear to be equivalent as the four NAD⁺ mean B factors are quite similar (41.2, 42.9, 43.1 and 42.5 Å²). As mentioned above, each dinucleotide molecule is well defined in the electron density map and adopts the extended conformation reported to be suitable for efficient hydride transfer. When compared with other known tetrameric structures of hydrolytic ALDHs, the greatest structural differences are observed in the nicotinamide and adenine binding pockets. First, the MSDH structure reveals a major difference in the stabilization mode of the nicotinamide ring that is well defined in the electron density maps, in contrast to what has been observed in nearly all x-ray structures of binary ALDH-NAD(P)⁺ complexes. This difference could originate from a stabilization of its conformation through hydrogen bonds between the carboxamide group and the carbonyl group of Thr-269 on the one hand and a water molecule held in position by the carbonyl group of Ser-471 and the side chain of Asn-427 on the other hand (Fig. 1). We, therefore, generated the N427L substitution to determine the consequence of the loss of this MSDH-specific hydrogen bond. This substitution dramatically altered the catalytic properties of the enzyme. The acylation became rate-limiting with a decrease of the associated rate constant by at least 10²-fold relative to the wild-type MSDH (i.e. <10⁻² versus 1000 s⁻¹). Although it was not possible to determine whether hydride transfer was rate-determining within the acylation step because of the very low kcat value, the dramatic decrease of the kcat is likely the consequence of the disruption of the H₂O-mediated hydrogen bond interaction between the carboxamide and the Asn-427, thus leading to formation of an inefficient N427L ternary complex.

By contrast, several interactions known to be essential in the hydrolytic ALDHs for maintaining a productive conformation of the NMN moiety are conserved. The NMN ribose remains anchored through hydrogen bonds between its hydroxyl groups and the side chain of the invariant Glu-399. Additionally, one of the two methyl groups of the β-branched side chain of Val-244 is positioned similarly to the β-methyl of invariant Thr-244 in hydrolytic ALDHs. Therefore, the methyl could fulfill a similar function as that of Thr-244 due to its positioning via the hydrophobic interaction of the second β-methyl with the side chain of Met-178. Altogether, these structural data suggest that the “classical” pattern of interactions proposed to participate in the stabilization of an efficient hydride transfer conformation of the NMN moiety of the cofactor, once the transient hemithioacetal intermediate is formed, is also operative in the MSDH, a result that is likely generalizable to CoA-dependent ALDHs. However, the contribution of Val-244 in stabilizing the nicotinamide conformation appears not sufficient, whereas the carboxamide/Asn-427 interaction is shown to be essential.

Interestingly, the pocket that is postulated to constitute the exit door of the reduced cofactor in the hydrolytic ALDHs (supplemental Fig. S3) and the interaction pattern that enables the pyrophosphate group to be pliable are conserved (data not shown). Therefore, a conformational isomerization of the NMN moiety is possible, but questions remain concerning its mechanistic relevance. In accordance with the ping-pong kinetic mechanism, NADH release occurs before the transthioesterification step. This rules out the requirement for a flip of the NMNH for CoA binding, an assertion that is further supported by our recent kinetic data showing that the NAD(H) and CoA binding sites do not overlap (23). An alternative explanation is that nucleophilic attack of the CoA on the decarboxylated thioacylenzyme intermediate is not possible if NMNH is present in the active site due to potential steric hindrance. Nevertheless, this hypothesis requires additional structural data to be validated, in particular characterization of the CoA binding site. As the putative structural dynamics associated with the NMN(H) moiety did not provide any clues as to the molecular basis responsible for the early release of the reduced cofactor in the catalytic mechanism of the MSDH, we investigated the possibility that this difference could originate from significant disparities in the adenosine binding mode.

Adenosine Binding Site—Superimposition of all available ALDH holo-structures revealed that the adenine ring is substantially more solvent-exposed in MSDH (Fig. 1). Analysis of the adenine binding pocket highlighted several structural factors that could explain this marked difference. The adenine ring fits between the N-terminal parts of the αf and αc helices, making van der Waals contacts with the side chains of His-226 and Val-229 from αf and of Val-249 from αc, respectively (supplemental Fig. S4A). The Val-249 residue is well conserved in all ALDHs including MSDHs, but His-226 and Val-229 are MSDH-specific (supplemental Figs. S2 and S5). The presence of invariant Val-229 in MSDH reduces the depth of the binding pocket, thereby increasing the solvent exposure of the adenine ring. In hydrolytic ALDHs, this residue is replaced by a glycine or an alanine, whereas the residue corresponding to His-226 is quite variable (except for eukaryotes where a proline is preferentially observed) (supplemental Fig. S2). In addition, the residue corresponding to Gly-225 is also missing, an invariant feature in MSDH sequences (supplemental Fig. S5). It was thus tempting to postulate that the adenine binding mode plays a key role in the dynamics of the reduced cofactor during the MSDH catalytic cycle. To validate this assumption, we first attempted to improve the stabilization of the adenine ring through substi-
tution of glycine for Val-229 and insertion of glycine at position 225 (::G225). If our hypothesis was correct, we expected to observe a significant decrease in the rate constant \( k_{\text{off}} \) associated with the dissociation of NADH from the thioacylenzyme/NADH complex relative to wild-type MSDH.

For the wild-type MSDH-catalyzed reaction, the rate-limiting step was shown to be associated with \( \beta \)-decarboxylation within the deacylation step. It was also demonstrated that the \( \beta \)-decarboxylation of the thioacylenzyme intermediate occurs after NADH release and before transthioesterification (22). Therefore, before interpreting the steady-state kinetic data and the \( k_{\text{off}} \) values, it was necessary to establish whether the rate-limiting step was still associated with deacylation for the mutated MSDHs. Accordingly, fast kinetic experiments were carried out for all mutated MSDHs at 30°C and pH 8.2 in the presence of saturating concentrations of NAD" and MMSA under presteady-state conditions (i.e. in the absence of CoA).

Kinetic Properties of Mutated V229G, ::G225 and V229G/::G225 MSDHs—A burst magnitude of 2 mol of NADH/mol of tetramer was observed irrespective of the mutated MSDHs. These data indicate that all mutated MSDHs also exhibited half-site reactivity, as described for the wild type. More importantly, the \( k_{\text{obs}} \) values of 152, 94, and 173 s\(^{-1}\) were 195-, 241-, and 824-fold higher than the \( k_{\text{cat}} \) values for the V229G, ::G225 and V229G/::G225 MSDHs, respectively. This result shows that the rate-limiting step still takes place after hydride transfer. Its efficiency remains high but is significantly decreased, as the \( k_{\text{obs}} \) values are 7- to 13-fold lower relative to wild-type MSDH. This drop in rate constant could be the consequence of minor changes occurring in the location and orientation of the nicotinamide relative to the hemithioacetal intermediate within the mutated ternary complexes, thereby slowing down hydride transfer. One possible explanation is that modifications in the adenine binding pocket initiated a cascade of structural changes that disturbed the nicotinamide positioning within the covalent ternary complex. Interestingly, this hypothesis is supported by the recent work of Tsybovsky and Krupenko (40), who proposed that long-range communication between the active site and the cofactor binding domain in ALDH1L1, involving an \( \alpha \)-helix (\( \alpha_\alpha \)) that forms one-half of the adenine binding pocket, partly controls the binding of the cofactor.

The kinetic parameters determined at pH 8.2 and 30°C under steady-state conditions are summarized in Table 2.

<table>
<thead>
<tr>
<th>( K_m ) values</th>
<th>NAD(^+)</th>
<th>MMSA</th>
<th>CoA</th>
<th>( k_{\text{cat}} )</th>
<th>Rate-limiting step</th>
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<tr>
<td>Wild type</td>
<td>2.3 ± 0.1</td>
<td>60 ± 10</td>
<td>120 ± 20</td>
<td>2.2 ± 0.2</td>
<td>Deacylation</td>
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<tr>
<td>V229G</td>
<td>0.69 ± 0.05</td>
<td>22 ± 4</td>
<td>63 ± 4</td>
<td>0.21 ± 0.01</td>
<td>Deacylation</td>
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<tr>
<td>::G225</td>
<td>0.15 ± 0.02</td>
<td>27 ± 4</td>
<td>119 ± 25</td>
<td>0.090 ± 0.007</td>
<td>Acylation</td>
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<tr>
<td>V229G/H226P</td>
<td>0.66 ± 0.19</td>
<td>28 ± 6</td>
<td>96 ± 23</td>
<td>0.020 ± 0.006</td>
<td>Acylation</td>
</tr>
<tr>
<td>V229G/::G225/H226P</td>
<td>0.77 ± 0.10</td>
<td>21 ± 5</td>
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<tr>
<td>V229G/::G225/V252I</td>
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<td>V229G/::G225/V253I</td>
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* Data are from Ref. 22.
Conclusions

Structural dynamics associated with cofactor binding are known to play key roles in the chemical mechanism of the hydrolytic ALDHs. In this study, we propose that this is the case for their CoA-dependent counterparts. Indeed, a flip of the NMNH away from the active site is also possible in hydrolytic ALDHs, the flip of the NMNH away from the active site is also possible in

FIGURE 2. Representative transient for the determination of the NADH dissociation rate from the V229G/::G225 thioacylenzyme intermediate.

The collected data were fit to a triphasic or biphasic expression for the wild-type (thick line, (22)) and V229G/::G225 (thin line) MSDHs, respectively. The first phase represents the acylation step, and the second phase represents the consumption of NADH after its release from the thioacylenzyme-ALDH complex, whereas the third phase observed for the wild-type MSDH is likely due to the reverse LDH-catalyzed reaction. For the V229G/::G225 MSDH, the rate constants obtained for the global fitting are 29 ± 1 and 1.0 ± 0.1 s⁻¹, respectively. The burst of NADH appears smaller for the wild-type enzyme because the rate constants associated with the two phases are very similar (72 versus 56 s⁻¹) compared with those determined for the mutated MSDH.

Cofactor Dynamics in ALDH Superfamily

The steady-state kinetic parameters determined at pH 8.2 and 30 °C are summarized in Table 2. The $k_{cat}$ values decreased from 24- to 110-fold. More importantly, the fact that no burst of NADH production was observed under pre-steady-state conditions shows that acylation is now rate-limiting irrespective of the mutated MSDH (this result precludes comparison of the obtained $K_m$ values with those determined for the wild type). As mentioned in the Introduction, the rate constant associated with the acylation step is high for the wild-type ($k_{ac} > 1000$ s⁻¹). Therefore, the $k_{ac}$ values are at least $1.1 \times 10^4$- to $5 \times 10^4$-fold lower for the mutated MSDHs. These drastic kinetic effects are likely the consequence of significant changes in the positioning of the nicotinamide ring relative to the hemithioacetal intermediate that prevent an efficient hydride transfer. Because the mutations should modify the positioning or mode of stabilization of the adenine, it is tempting to propose that changes occurring in this binding pocket can propagate to the nicotinamide ring. Although this hypothesis remains to be validated, our data clearly indicate that the cofactor binding mode is optimized in MSDH with a fine balance between the requirement for positioning of the nicotinamide ring to allow efficient hydride transfer and a degree of conformational flexibility for the deacylation to occur. Any perturbation, even minor, appears to be deleterious with respect to the efficiency of the MSDH-catalyzed reaction. The model seems to hold for the hydrolytic ALDHs. Indeed, our attempts to accelerate the rate of NADH release through "destabilization" of the adenine ring failed for retinal dehydrogenase 2 (RALDH2). The NMN moiety is known to be less stabilized in this ALDH family, at least within the ALDH/NAD(P) binary complex. Therefore, if the main anchor point (i.e., the adenine ring) is weakened, it is likely that the positioning of the NMN moiety within the covalent ternary complex is compromised, explaining why the ΔG225/G229V RALDH2 acylation becomes rate-limiting with a 300-fold reduced $k_{ac}$ (data not shown).

Conclusion—Structural dynamics associated with cofactor binding are known to play key roles in the chemical mechanism of the hydrolytic ALDHs. In this study we propose that this is also the case for their CoA-dependent counterparts. Indeed, a flip of the NMNH away from the active site is also possible in MSDH and likely the entire CoA-dependent family. This isomerization step might be required to permit nucleophilic attack of the CoA on the decarboxylated thioacylzyme. However, in contrast to what has been established for hydrolytic ALDHs, the flip of the NMNH away from the active site leads to the complete release of NADH. Our data show that weaker stabilization of the adenine ring is a key factor in triggering the early NADH release in the MSDH-catalyzed reaction and provide for the first time structural insights into the mechanism whereby the cofactor binding mode is responsible, at least in part, for the different kinetic behaviors of the hydrolytic and CoA-dependent ALDHs. From similar three-dimensional structures, evolution has thus produced slightly different modes of cofactor binding within the ALDH superfamily. Over-

this insertion instead increased the length of the loop located at the N-terminal end of the αC helix. Thus, it is reasonable to postulate that the 56-fold decrease in $k_{off}$ for NADH results from a conformational change(s) of this loop that might provide a supplemental anchor point(s) that improves the stabilization of the adenine ring. However, although each single mutation led to significant kinetic effects, they were not additive, as no additional decrease in $k_{off}$ was observed for the double mutant. Finally, the fact that the $k_{cat}$ values are only 2.5- to 5-fold higher than the $k_{cat}$ values shows that β-decarboxylation likely remains rate-limiting irrespective of the mutated MSDHs. Nonetheless, the decrease in $k_{cat}$ compared with the wild-type MSDH is mainly the consequence of the significant decrease in the $k_{cat}$ values. Therefore, NADH release can be considered to be partially rate-limiting.

Kinetic Properties of Mutated V229G/H226P, V229G/::G225/H226P, and V229G::G225/V252L/V253I MSDHs—In an attempt to make NADH release rate-limiting, we tried to further stabilize the adenine ring by combining new point mutations with V229G and ::G225. As mentioned above, the data obtained on ::G225 MSDH highlight the fact that mutations altering the conformation of the loop located at the entrance of the binding pocket can lead to improved stabilization of the adenine ring. To validate this assumption, a proline was introduced at position 226 in the V229G and V229G/::G225 MSDHs. Substitution of a proline for His-226 mimics the molecular context of many hydrolytic ALDHs and introduces strong geometrical constraints that should modify the conformation of this loop. Therefore, we anticipated that the H226P substitution would induce a closure, even minor, of this loop on the adenine ring. On the other hand, introduction of residues with higher hydrophobic character, such as Leu and Ile, at positions 252 and 253 (αC helix, supplemental Fig. S4) in V229G/::G225 MSDH, would potentially increase the number of van der Waals contacts between adenine and the bottom face of the binding pocket.

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Cofactor Dynamics in ALDH Superfamily

all stabilization of the cofactor results from differential contributions of both binding subsites of the cofactor; that is, mainly interactions with the adenine moiety for the hydrolytic ALDHs and the nicotinamide for the CoA-dependent ALDHs. However, in both cases the nicotinamide ring is properly positioned in the ternary complex to allow an efficient hydride transfer during the acylation step, whereas differences in the stabilization of the adenine ring lead to early or late release of the reduced cofactor.

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