10-FORMYLTERAHYDROFOLATE DEHYDROGENASE REQUIRES 4'-PHOSPHOPANTETHEINE PROSTHETIC GROUP FOR CATALYSIS*

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Running title: FDH is a 4'-phosphopantetheine requiring enzyme

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10-Formyltetrahydrofolate dehydrogenase (FDH) consists of two independent catalytic domains, amino and carboxyl-terminal, connected by a 100 amino acid residue linker (intermediate domain). Our previous studies on structural organization and enzymatic properties of rat FDH suggest that the overall enzyme reaction, that is an NADP⁺-dependent conversion of 10-formyltetrahydrofolate to tetrahydrofolate and CO₂, consists of two steps: (i) hydrolytic cleavage of the formyl group in the amino-terminal catalytic domain followed by (ii) NADP⁺-dependent oxidation of the formyl to CO₂ in the carboxyl-terminal aldehyde dehydrogenase domain. In this mechanism, it was not clear how the formyl group is transferred between the two catalytic domains after the first step. The present studies demonstrate that the intermediate domain functions similarly to an acyl carrier protein. A 4'-phosphopantetheine swinging arm, bound through a phosphoester bond to Ser354 of the intermediate domain, transfers the formyl group between catalytic domains of FDH. Thus, our studies define the intermediate domain of FDH as a novel carrier protein and provide the previously lacking component of the FDH catalytic mechanism.

The subunit of 10-formyltetrahydrofolate dehydrogenase (FDH or Aldh1L1, EC 1.5.1.6) represents a single polypeptide consisting of three domains, with two of these domains possessing their own catalytic activities (1-4). The amino-terminal domain of FDH (residues 1-310) functions as 10-formyltetrahydrofolate (10-fTHF) hydrolase converting 10-fTHF to tetrahydrofolate (THF) and formate (Fig. 1). The carboxyl-terminal domain (residues 420-902) is an aldehyde dehydrogenase-homologous enzyme capable of NADP⁺-dependent oxidation of short-chain aldehydes to corresponding acids (3). The two catalytic domains are separated by a short, about a hundred residues, linker domain (the intermediate domain). While the physiological significance of the catalytic functions of the separate domains of FDH is not clear, the well-established function of FDH is the conversion of 10-fTHF to THF in a NADP⁺-dependent dehydrogenase reaction (5-7). It is believed that this reaction regulates intracellular 10-fTHF/THF pools (8), controls de novo purine biosynthesis (9,10) and affects methylation potential of the cell (11). This reaction is only observed when the two catalytic domains are linked in one polypeptide by the intermediate domain (2).

Studies of FDH structure and catalytic mechanism (12-18) suggest that this reaction proceeds in two steps: (i) the hydrolytic removal of formyl group from 10-fTHF and (ii) a NADP⁺-dependent oxidation of formyl to CO₂. The first step takes place in the amino-terminal hydrolase domain while the second step occurs in the carboxyl-terminal aldehyde dehydrogenase domain (2,3,17,18).

The connection between the two steps is the transfer of the formyl group between the two catalytic domains. The nature of such transfer, however, is not obvious. We have previously demonstrated that the two catalytic domains of FDH do not form close contacts in the absence of the intermediate domain (2). It has been suggested that the intermediate domain keeps the amino- and carboxyl-
terminal domains of FDH in close proximity to each other, and in the correct orientation, to create an interface between the two domains and to allow the formyl group transfer between the two catalytic centers. In support of this, engineering of FDH with limited flexibility within the intermediate domain, that presumably permanently uncouples the two catalytic domains, resulted in the enzyme with no 10-fTHF dehydrogenase activity (15). The crystal structures of the two domains, however, have demonstrated that the catalytic centers of FDH, the hydrolase and aldehyde dehydrogenase, are buried within their corresponding domains so that residues of one catalytic center can not contact residues within the other catalytic center (17,18). This implies that the direct transfer of formyl group between the two centers is improbable and suggests a role for the intermediate domain in the transfer of the substrate between the catalytic centers.

Several mechanisms for channeling of a substrate within an FDH molecule could be envisioned. If the formyl group moves from the hydrolase catalytic center into the aldehyde dehydrogenase catalytic center by passive diffusion, the role of the intermediate domain could be in maintaining an orientation of the two centers that cages formyl and minimizes its loss to the outer solution. Another potential mechanism by which the intermediate domain can facilitate the transfer of a reaction intermediate between the catalytic centers is formation of a molecular tunnel, as has been proposed for a number of multidomain enzymes (reviewed in (19,20)). The fact that such tunnels are mostly hydrophobic in their nature (20) while the intermediate domain of FDH is noticeably hydrophilic (15) argues against such mechanism. A third alternative for assisted intermediate transfer involves the employment of a prosthetic group, which can function as a swinging arm (21). The canonical examples of such transfer are pyruvate carboxylase (22), the pyruvate dehydrogenase complex (23) and acyl carrier proteins (ACP) (24) that use biotine, lipoate and 4'-phosphopantetheine (4'-PP), correspondingly, as prosthetic groups.

The NMR structure of the intermediate domain of human FDH (RCSB entry 2CQ8) demonstrates an ACP-like fold (21). In the present study we have evaluated the sequence and structure similarity between the intermediate domain of rat FDH and carrier proteins with a 4'-PP prosthetic group (Fig. 2). We also provide evidence that the intermediate domain of rat FDH is covalently modified by 4'-PP and that this prosthetic group transfers formyl from the hydrolase catalytic center to the aldehyde dehydrogenase catalytic center during the FDH-catalyzed 10-fTHF dehydrogenase reaction.

**EXPERIMENTAL PROCEDURES**

**Protein expression and purification** – Rat FDH was expressed either in *E. coli* or in insect cells using a baculovirus expression system as we previously described (18,25). It has been purified using affinity chromatography on immobilized 5-formyl-THF Sepharose followed by size-exclusion chromatography on Sepharcl S300. If necessary, additional purification has been done by FPLC using a Mono Q column as described (25). The S354A mutant of FDH was expressed in insect cells and purified by the same procedure as the wild type enzyme. The intermediate domain of rat FDH has been expressed in *E. coli* and purified from inclusion bodies using size exclusion chromatography on Sephacryl S300 (15).

**Assay of enzyme activity** – Hydrolase, aldehyde dehydrogenase and 10-fTHF dehydrogenase activities of FDH were evaluated spectrophotometrically as previously described (3). Briefly, the hydrolase and 10-fTHF dehydrogenase activities were measured using 10-formyldeazafolate as a substrate, with the assays for the dehydrogenase activity containing additionally 100 µM NADP⁺. The monitored increase in absorbance at 295 nm (the characteristic spectrum maximum for the reaction product, dideazafolate) was used to calculate the specific activity. All assays were carried out at 30 °C in a 1-cm quartz cuvette using Shimadzu 2401PC double-beam spectrophotometer. Aldehyde dehydrogenase activity was evaluated by assaying propanal...
oxidation in the presence of FDH and NADP⁺. The reduction of NADP⁺ to NADPH measured at 340 nm (the characteristic spectrum maximum for the reduced form of the coenzyme) provided the reaction rate for the aldehyde oxidation.

Site-directed mutagenesis – The S354A mutant of rat FDH was generated using Quick Change kit (Stratagene) as we previously described (13,14).

Re-activation of E. coli expressed FDH – Purified recombinant rat FDH expressed in E. coli (50 µl of 2 mg/ml solution in 20 mM Tris-HCl buffer pH 7.5) was incubated with 2 µl of 5 mM coenzyme A solution, in the presence and in the absence of 50 or 100 µl of lysate from Sf9 insect cells. The cell lysate was obtained using mechanical disruption of a suspension (1.0 ml) of 10 x 10⁶ cells in 20 mM Tris-HCl buffer pH 7.5 containing 10 mM MgCl₂, 10 mM DTT and 0.1 mM PMSF using Dounce homogenizer, for 2 min. Then 200 µl of glycerol was added to the homogenate and insoluble content was removed by centrifugation (13,000 g, 5 min).

Synthesis of fluorescent reporter – Fluorescent reporter was synthesized according to a published procedure (26). One hundred µl of fluorescein maleimide (7 mg in 0.7 ml of DMSO) solution was mixed with 2 ml 0.1 M of MES buffer pH 6.0 containing 3.8 mg of coenzyme A and kept on ice for 30 min followed by incubation for 10 min at room temperature. Complete conversion of the coenzyme A into fluorescent reporter was verified by TLC (butanol:acetic acid:water at v/v ratio 5:2:4). Non-reacted fluorescein maleimide was quenched by addition of 40 µL of 0.5 M DTT in water. The solution was used directly for protein labeling. It was stable upon freezing for at least a month.

Labeling FDH with the fluorescent reporter – Fifty µl of purified FDH (2.0 mg/ml solution in 20 mM Tris-HCl buffer pH 7.8) was incubated with 50 or 100 µl of lysate from Sf9 cells and 10 µl of the fluorescent reporter mixture for 3 h at 37 °C. Following incubation 10 µl of this solution was mixed with 10 µl of SDS-PAGE loading buffer (4% SDS, 1% 2-mercaptoethanol, 4% glycerol and 0.01% bromochlorophenol blue in 50 mM Tris-HCl pH 6.8) and subjected to SDS-PAGE. Immediately following electrophoresis the gel was imaged under UV irradiation and then stained with Coomassie Blue dye.

Labeling FDH with fluorescein maleimide – One µg of FDH in 1 ml of phosphate buffer, pH 7.0 was incubated with 25 µl of the stock solution of fluorescein maleimide (Pierce) in DMSO (20 mg/ml) at room temperature for 3 h. Non-reacted fluorescein maleimide was quenched by addition of 25 µl of 100 mM DTT and removed by gel filtration on PD10 column (Pharmacia).

Treatment with alkaline phosphatase – Purified wild-type FDH or its S354A mutant (about 1 mg) were incubated with 20 units of CIAP (calf intestinal alkaline phosphatase, New England Biolabs) in 50 mM Tris-HCl pH 7.9 containing 100 mM NaCl, 10 mM MgCl₂ and 1 mM DTT, at 37 °C for 24 h. The protein component was then separated by filtration on Centriprep cartridge with molecular mass cutoff of 3,000 Da (Millipore). The treated FDH was evaluated for all three catalytic activities as described above. The low molecular mass filtrate was concentrated and subjected to HPLC on C18 column.

HPLC – The concentrated low molecular mass filtrate was diluted with 20 mM ammonium acetate buffer pH 6.8 (1:1 ratio) and 20 µl was loaded on a Microsorb-MV C18 column (4.6x250 mm, 5-µm-diam. particles, Rainin), equilibrated with the same buffer. Elution was performed with a linear gradient of acetonitrile (0-60%) in the above buffer over 60 min. Chromatography was carried out on a Waters 515 dual-pump solvent delivery system at a flow rate of 1 ml/min; peaks were monitored at 224 nm using Waters 2487 dual-wavelength absorbance detector. Fractions of 1 ml were collected.

MALDI mass-spectrometry – Fractions corresponding to peaks observed at HPLC separation were analyzed using matrix assisted laser desorption ionization mass spectrometry (MALDI-MS). A time of flight MALDI Altoflex Smartbeam instrument (Bruker Daltonics, Billerica, MA) was used in
linear mode to detect average ion masses. Samples were prepared by mixing 1 part of a sample solution (0.5 µl) with 1 part of matrix (5 mg/ml 2,5-dihydroxybenzoic acid in 50% ethanol) on the sample plate and allowed to air dry prior to analysis. Experiments were performed at Biomolecular Mass Spectrometry Facility of MUSC.

RESULTS

FDH treated with an alkaline phosphatase lacks 10-formylTHF dehydrogenase activity – 4’-PP is bound to ACP by a phosphodiester bond, which can be hydrolyzed by the action of a phosphodiesterase resulting in the removal of 4’-PP group (27). To study whether the FDH intermediate domain has the same type of modification as ACPs, we have treated the fully active rat FDH, expressed in insect cells, with CIAP. Alkaline phosphatases in general have been shown to possess phosphodiesterase activity (28-30). Therefore, we suggested that CIAP is also capable of such an activity and will hydrolyze the phosphodiester bond between FDH and 4’-PP. Indeed, we observed that the treatment with alkaline phosphatase completely deprived FDH of 10-fTHF dehydrogenase activity while the treated enzyme was still capable of 10-fTHF hydrolase activity and aldehyde dehydrogenase activity (Table 1).

Site-directed mutagenesis of Ser354 – Comparison of the sequences of the intermediate domain of FDH and ACPs indicates that Ser354 is equivalent to the serine residue of ACPs that is modified by 4’-PP (Fig. 2A). To study the role of this residue in FDH function we have expressed the S354A mutant enzyme. The mutant enzyme was expressed in insect cells using a baculovirus expression system and purified to near homogeneity by the same procedure as the wild type enzyme (16). Assay of activities of the mutant enzyme revealed that it possesses the hydrolase and aldehyde dehydrogenase activities similar to those of the wild type FDH (Table 1). In contrast, the mutant did not produce noticeable level of the 10-fTHF dehydrogenase activity (Table 1).

Re-activation of E. coli expressed FDH - We have previously observed that E. coli expressed rat FDH lacks the dehydrogenase activity towards 10-fTHF while the two other activities, hydrolase and aldehyde dehydrogenase, residing in specific domains, remained (unpublished data). These results were initially explained by a lack of ability of E. coli to fold FDH into a functional enzyme. However, if FDH requires a modification of its intermediate domain, it is possible that the enzyme expressed in bacteria is inactive due to the lack of specific enzymes in E. coli capable of modifying FDH with 4’-PP. This was found to be the case: incubation of E. coli expressed FDH with lysates of insect cells, in the presence of CoA (CoA is a source of 4’-PP for ACP (24)), restored the 10-fTHF dehydrogenase activity (Table 1). Similar results were obtained in experiments with lysates from human A549 cells (data not shown). To confirm that activation of FDH proceeds through a modification of Ser354, we have expressed the S354A FDH mutant in E. coli and subjected it to the same procedure. These experiments revealed that this mutant was not reactivated into a functional 10-fTHF dehydrogenase (Table 1).

Fluorescent labeling of FDH - Further evidence of the covalent modification of FDH at Ser354 in the presence of CoA came from our experiments with a fluorescent reporter synthesized according to a published procedure (26). In this reporter, the fluorescent label, fluorescein maleimide, was attached to a 4’-PP part of CoA through the sulfhydryl group (Fig. 3A). This reporter allows fluorescent labeling of proteins undergoing 4’-PP modification as illustrated in Fig. 3B (26). We have incubated purified recombinant FDH or its intermediate domain expressed in E. coli (both presumably unmodified at Ser354), with the reporter and lysates from Sf9 insect cells and subjected the mixture to SDS-PAGE. These experiments clearly demonstrated incorporation of the fluorescent label into both FDH and its intermediate domain (Fig. 3, C and F). In contrast, the S354A mutant was not labeled under the same conditions indicating that this serine is a site for the modification (Fig. 3D). We have further used insect cell-
expressed FDH, possessing 10-THF dehydrogenase activity, as a target for modification by this reporter. We failed to observe modification of FDH with fluorescently labeled 4’-PP (Fig. 3E) presumably because the enzyme is already fully modified with 4’-PP.

We also used fluorescein maleimide to modify sulfhydryl groups of insect cell expressed FDH. Since this enzyme is a functional 10-THF dehydrogenase, we expect that it bears 4’-PP prosthetic group with a sulfhydryl group accessible for modification. The labeled protein, separated from non-reacted fluorescein maleimide, was then treated with CIAP to release components bound to the protein through a phosphodiester bond. The released low molecular mass components were then separated from the protein by passing through a 3,000 Da cutoff filtration cartridge. In a control experiment, labeled protein untreated with CIAP was subjected to the same procedure. We have observed strong fluorescence of the filtrate in the case of phosphatase-treated FDH while no fluorescence was seen in the control, non-treated sample. In contrast, the S354A FDH mutant did not produce labeled low molecular mass components in similar experiments. This further confirmed that a sulfhydryl-containing cofactor is bound to Ser354 of FDH through a phosphodiester bond.

**Mass-spectrometry analysis of FDH modification** – We have analyzed the molecular mass of the modifying group of FDH by MALDI TOF mass-spectrometry. The fact that treatment with CIAP completely deprived the enzyme of 10-THF dehydrogenase activity indicates that the essential non-protein cofactor was removed from the protein core by this treatment. We have separated the cofactor from the protein moiety by concentrating the mixture on a filter with 3,000 Da molecular mass cutoff and subjected the low molecular mass content, passing through the filter, to HPLC on a C18 column. The chromatography produced two major peaks with the retention time 17.5 and 49 min, correspondingly. The MALDI TOF analysis has revealed that the main specie in the first peak has molecular mass of 550.60 Da while the main component of the second peak has the molecular mass of 360.08 Da. The molecular mass of the latter corresponds well to the calculated molecular mass of 4’-PP, 358.33 Da. As for the first peak, we suggest that its main component represents an oxidized form (dimerized via a disulfide bond) of the two pantetheine residues, produced by removal of phosphate from 4’-PP by CIAP. The calculated molecular mass of such a dimer is 552 Da. As a control, both E. coli expressed FDH and the S354A mutant expressed in insect cells were subjected to the same procedure. None of the proteins yielded corresponding peaks after HPLC (data not shown).

**DISCUSSION**

The amino acid sequence of FDH suggests that the enzyme is a natural fusion of at least two unrelated genes. While the relationships of the amino-terminal domain with other enzymes that use 10-THF as a substrate, as well as the nature of the carboxyl-terminal domain as an aldehyde dehydrogenase family member were apparent, the origin of the linker between the two domains remained unclear. Indeed, this domain does not have an extensive sequence similarity to known proteins. However, recent analysis of the sequence of rat FDH using a database of protein domains (www.expasy.org/prosite) suggests that the intermediate domain is a member of the group of carrier proteins with a 4’-PP swinging arm.

These carrier proteins are components of multienzyme complexes involved in fatty acid, polyketide and non-ribosomal peptide biosynthesis (21,31). Their specific feature is a 4’-PP component, covalently attached to a conserved serine residue through a phosphodiester bond (24). Another enzyme with a 4’-PP modification is α-aminoadipate reductase, a component of lysine synthesis specific to fungi (32). The 4’-PP arm is a crucial functional component of the carrier proteins: it holds a growing chain of fatty acids, polyketides or peptides during the reaction. In mammals, there is only one known metabolic pathway, fatty acid biosynthesis, which requires this type of carrier proteins.
Structures of several carrier proteins have been solved (reviewed in (21,24,31)). They demonstrate a similar fold, a distorted four-helix bundle with the 4'-PP bearing conserved serine located in the loop. The NMR structure of the intermediate domain of human FDH has recently become available (PDB entry 2CQ8). The model of the rat enzyme, which was used in our studies, was built using the human enzyme as a template. The overall fold of the intermediate domain is similar to that of other carrier proteins (33-35) with the best superposition (r.m.s.d. between Ca atoms 4.1 Å) with the peptidyl carrier domain of nonribosomal tyrocidine synthase III from B. brevis (35) (Fig. 2B). Most of the structural deviations among the carrier proteins are due to shifted positions of the four α-helixes within each structure, as well as alterations in the length and conformation of the loops connecting the α-helixes. But the position of the conserved serine residue that bears the 4'-PP arm is very close in all structures (Fig. 2B).

Interestingly, despite the overall structural similarity, the amino acid sequence conservation within this group is not so profound: the multiple sequence alignment of the four carrier proteins from different pathways (non-ribosomal peptide synthase, polyketide synthase, ACP and the intermediate domain) revealed only two strictly conserved residues, other than the serine modified by 4'-PP.

The present study has demonstrated that the intermediate domain of FDH is a functional analog of carrier proteins with the 4'-PP arm. This prosthetic group must be attached to the conserved serine residue (Ser354) in order for FDH to be catalytically active. Elimination of this modification (in our studies via replacement of the serine with alanine) resulted in FDH lacking 10-fTHF dehydrogenase activity. Furthermore, the treatment of active FDH with alkaline phosphatase deprived the enzyme of 10-fTHF dehydrogenase activity. Such a treatment also yielded a compound with a molecular mass that corresponds to the molecular mass of 4'-PP. To complement these experiments, incubation of catalytically inactive FDH, expressed in E. coli, with lysates of mammalian or insect cells and CoA as a source of 4'-PP, restored the enzyme activity. Incorporation of 4'-PP into FDH as well as into the intermediate domain itself was further confirmed in our in vitro experiments with a fluorescent reporter. Thus, it becomes apparent that the intermediate domain uses a 4'-PP arm to transfer a formyl group between the two catalytic centers (Fig. 5).

Atomic level details concerning the interface between FDH domains and the nature of their interaction with the 4'-PP swinging arm await the crystal structure of the full length FDH. But since the 4'-PP arm extends to about 20 Å in length (21), both the hydrolase and aldehyde dehydrogenase catalytic centers of FDH are well within reach as can be concluded from their respective crystal structures (17,18,36). The catalytic center of the hydrolase domain is relatively open (17,36) that perhaps facilitates easy access of the 4'-PP arm to the formyl group of the substrate. Moreover, two potential mechanisms could further facilitate access of the 4'-PP arm to the formyl group in the hydrolase catalytic center. In one of the mechanisms, a shift of the flexible loop, bearing catalytic Asp142, could expose formyl to the domain surface (17). Another mechanism may involve larger scale rearrangements within the hydrolase domain making the catalytic center cleft more open (36). In contrast, the aldehyde dehydrogenase active site is less accessible, with its key catalytic cysteine located at the end of a 12 Å deep substrate entrance tunnel (18). The length of the 4'-PP arm, however, is sufficient to bring the formyl group in close vicinity of this residue. Interestingly, the substrate entrance tunnel of the aldehyde dehydrogenase domain of FDH shares a characteristic with aldehyde dehydrogenases specific for larger aldehyde substrates namely a wide tunnel made up of amino acids with small side chains (37,38). Apparently, such a wide tunnel is necessary to allow the access of 4'-PP arm into the active site.

Our studies have answered the previously unclear question of how the two functional domains of FDH communicate to merge the hydrolase and aldehyde
dehydrogenase catalytic engines into one mechanism (Fig. 5). Importantly, this study has identified the second metabolic pathway in higher organisms, in addition to fatty acid biosynthesis, which uses the $4'$-PP prosthetic group. Interestingly, the two pathways, fatty acid biosynthesis and folate metabolism, are not closely related. The present finding also indicates that FDH requires post-translational modification to become a functional enzyme. A recently reported human $4'$-PP transferase with broad specificity (39) could be a candidate to carry out this modification (it appears that there is only one $4'$-PP transferase gene in the human genome (40)). Interestingly, FDH expressed in E. coli appeared to be unmodified although E. coli possesses several $4'$-PP transferases (41). These results might indicate that these transferases are not efficient towards large mammalian proteins. Alternatively, the lack of a typical consensus motif for $4'$-PP attachment can make FDH an inefficient substrate. Indeed, the corresponding sequence of rat FDH (Fig. 2B) indicates mismatches with the consensus motif (41,42), including two residues immediately up and downstream of the conserved serine. A third possibility to be considered is the activity of E. coli phosphodiesterase that removes $4'$-PP moiety from proteins (27).

Post-translational addition of $4'$-PP arm to ACP occurs after the protein is fully folded (41). Folded but unmodified FDH may occur if the modification process becomes deficient. Alternatively, hydrolytic enzymes, similar to a recently characterized ACP phosphodiesterase (27), may also alter the extent of phosphopantetheinylation of FDH in the cell. Whatever the mechanism, FDH without its $4'$-PP prosthetic group could function as a folate binding protein. Interestingly, the folate binding function was originally proposed for FDH (43). It is not clear at present whether FDH exists in the cell in unmodified form. If it does, the distribution between catalytically functional and non-functional FDH, and the cellular conditions that affect this distribution, would be of interest. Future studies should allow clarification of this matter.

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Abbreviations used are: 4′-PP, 4′-phosphopantetheine; 10-fTHF, 10-formyltetrahydrofolate; ACP, acyl carrier protein; CIAP, calf intestinal alkaline phosphatase; FDH, 10-formyltetrahydrofolate dehydrogenase; THF, tetrahydrofolate.

FIGURE LEGENDS

Fig. 1. Domain structure and catalytic activities of FDH. The amino-terminal domain of FDH produces hydrolase activity (converts 10-fTHF to THF and formate); the carboxyl-terminal domain produces aldehyde dehydrogenase activity (NADP⁺-dependent conversion of short chain aldehydes to corresponding acids); the full length FDH carries out NADP⁺-dependent conversion of 10-fTHF to THF and CO₂ (the other two reactions also remain).

Fig. 2. Sequence and structure similarity between intermediate domain of rat FDH and carrier proteins with 4′-PP modification. (A) Sequence alignments between the intermediate domain of rat FDH and carrier proteins (non-ribosomal peptide synthase (NRPS), polyketide synthase (PKS) or ACP). Bars represent α-helixes. Three strictly conserved residues between all four proteins (including 4′-PP modified serine) are shown by asterisks. (B) Superposition of the model of the intermediate domain of rat FDH (residues 317-398, green) onto structures of the PCP domain of the nonribosomal tyrocidine synthase III from B. brevis (PDB 1DNY (35), left panel), the actinorhodin polyketide synthase ACP from Streptomyces coelicolor (PDB 1AF8 (34), middle panel), and the fatty acid synthase ACP from E. coli (PDB 1ACP (33), right panel). The root mean square deviations between Ca atoms are 4.1, 5.5 and 8.6 Å, respectively. The model of the rat FDH intermediate domain was generated with the SWISS MODEL server (44) using the NMR structure of human FDH intermediate domain (PDB 2CQ8, 90.1% sequence identity) as the homology model. The structural superposition was performed using the SuperPose Version 1.0 server (45). The figure was prepared using Pymol (pymol.sourceforge.net).

Fig. 3. Fluorescent labeling of FDH at the putative 4′-PP modification site. (A) Structure of synthesized reporter for fluorescent labeling of FDH (fluorescein maleimide is attached to the sulfhydryl of CoA; the 4′-PP moiety is shaded). (B) Schematic depicting of fluorescent labeling of FDH by the reporter. (C) SDS-PAGE of E. coli expressed recombinant FDH incubated with the fluorescent reporter in the absence or in the presence of lysate from mammalian cells. Left panel, Coomassie stained gel; right panel, fluorescence of the same gel (the image after UV light irradiation). Two different concentrations of cell lysate were used in these experiments. (D), (E) and (F) In the experiments similar to (C) the insect cell expressed recombinant FDH, S354A mutant FDH or E. coli expressed recombinant intermediate domain, correspondingly were used. A single concentration of cell lysate was used in these experiments.

Fig. 4. Identification of FDH modification by MALDI TOF mass-spectrometry. (A) Separation of non-protein components, released from FDH after alkaline phosphatase treatment, by HPLC on C-18 column. (B) and (C), MALDI mass-spectrometry analysis of the peak 1 (retention time 17.5 min) and peak 2 (retention time 49 min) from (A), correspondingly.

Fig. 5. Proposed function of the intermediate domain in FDH mechanism. After the amino-terminal hydrolase domain removes formyl group from the substrate, 10-fTHF, the formyl becomes covalently attached to SH group of 4′-PP arm of the intermediate domain (step 1); the formyl-loaded arm swings from the amino-terminal hydrolase domain into the carboxyl-terminal aldehyde dehydrogenase domain (step 2); formyl transfers from 4′-PP to catalytic Cys707 in the aldehyde dehydrogenase catalytic center (step 3), where the reaction will be completed. Arrows shows transfer of formyl group.
Table 1. **Activity of FDH***

| Enzyme          | DH activity | Hydrolase activity | ALDH activity |
|-----------------|-------------|--------------------|---------------|
| wtFDH           | 100         | 100                | 100           |
| wtFDH/AP        | ND**        | 56                 | 74            |
| S354A           | ND          | 97                 | 101           |
| S354A (restored)| ND          | 90                 | 94            |
| wtFDH/E. coli   | ND          | 98                 | 95            |
| wtFDH/E. coli (restored) | 78       | 91                 | 92            |

*DH, 10-fTHF dehydrogenase; ALDH, aldehyde dehydrogenase. Activity is shown as percent to the corresponding activity of recombinant wild type FDH expressed in insect cells (wtFDH). AP, wtFDH treated with CIAP; wtFDH/E. coli, recombinant FDH expressed in *E. coli*. Restored, FDH was incubated with lysate from Sf9 cells in the presence of coenzyme A. The S354A mutant was expressed in insect cells.

**ND, not detectable.
Fig. 1
Fig. 2

A

IntD vs. NRPS (1DNY)

IntD vs. PKS (1AF8)

IntD vs. APC (1ACP)

B

Ser354

Ser 38

Ser 42

IntD vs. NRPS (1DNY)

IntD vs. PKS (1AF8)

IntD vs. APC (1ACP)
Fig. 3

A

Reporter: fluorescein maleimide

4'-phosphopantetheine

Coenzyme A

Coomassie UV FDH (E. coli)

FDH

Lysate

FDH

Lysate

FDH

S354A

FDH

S354A

IntD

IntD

D

Coomassie UV FDH Lysate

FDH Lysate

FDH Lysate

FDH

E

Coomassie UV FDH Lysate

FDH Lysate

FDH Lysate

FDH

F

Coomassie UV IntD Lysate

IntD

IntD

OH

Ser354

FDH

3'-P-AMP

Ser354

FDH

3'-P-AMP
Fig. 4

A

Absorbance, 224nm

Elution time, min

B

Intensity, [a.u.] x 10^4

m/z

C

Intensity, [a.u.] x 10^4

m/z
Fig. 5
10-formyltetrahydrofolate dehydrogenase requires 4'-phosphopantetheine prosthetic group for catalysis

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