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Characterization of a Fatty Acyl-CoA Reductase from *Marinobacter aquaeolei* VT8: A Bacterial Enzyme Catalyzing the Reduction of Fatty Acyl-CoA to Fatty Alcohol

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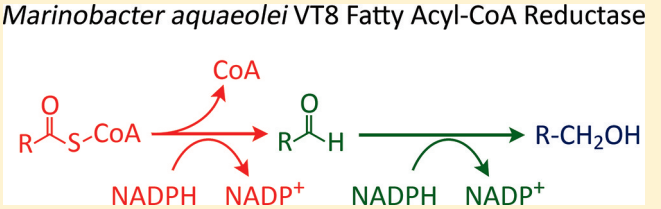
Supporting Information

ABSTRACT: Fatty alcohols are of interest as a renewable feedstock to replace petroleum compounds used as fuels, in cosmetics, and in pharmaceuticals. One biological approach to the production of fatty alcohols involves the sequential action of two bacterial enzymes: (i) reduction of a fatty acyl-CoA to the corresponding fatty aldehyde catalyzed by a fatty acyl-CoA reductase, followed by (ii) reduction of the fatty aldehyde to the corresponding fatty alcohol catalyzed by a fatty aldehyde reductase. Here, we identify, purify, and characterize a novel bacterial enzyme from *Marinobacter aquaeolei* VT8 that catalyzes the reduction of fatty acyl-CoA by four electrons to the corresponding fatty alcohol, eliminating the need for a separate fatty aldehyde reductase. The enzyme is shown to reduce fatty acyl-CoAs ranging from C8:0 to C20:4 to the corresponding fatty alcohols, with the highest rate found for palmitoyl-CoA (C16:0). The dependence of the rate of reduction of palmitoyl-CoA on substrate concentration was cooperative, with an apparent $K_m \sim 4\ \mu M$, $V_{max} \sim 200$ nmol NADP$^+$ min^{-1} (mg protein)$^{-1}$, and $n \sim 3$. The enzyme also reduced a range of fatty aldehydes with decanal having the highest activity. The substrate *cis*-11-hexadecenal was reduced in a cooperative manner with an apparent K_m of ~50 μM, V_{max} of ~8 μmol NADP$^+$ min^{-1} (mg protein)$^{-1}$, and $n \sim 2$.



Long chain fatty alcohols are widely used in cosmetics and soaps and are of interest as biofuels to substitute for petroleum-derived compounds.[1−4] Fatty alcohols are produced biologically in plants where they are used as components of the plant cuticle and in bacteria where they are condensed with fatty acids to make wax esters that function as energy storage compounds. In many plants, fatty acyl-CoAs are reduced to the corresponding alcohol either by two consecutive reduction steps (two electrons each) with the formation of the fatty aldehyde intermediate or by a single four-electron reduction (Figure 1). In pea leaves (*Pisum sativum* L.), two distinct fatty acyl-CoA reductases (FACoAR) have been identified, with one catalyzing the four-electron reduction to the fatty alcohol and the other the two-electron reduction to the fatty aldehyde.[5] In the jojoba plant and *Arabidopsis thaliana*, FACoAR enzymes have been reported that are capable of the four-electron reduction of a long chain fatty acyl-CoA directly to the corresponding alcohol.[6,7]

In contrast to the four-electron reductions catalyzed by several plant FACoAR enzymes, the FACoAR enzyme reported for the green algae *Botryococcus braunii* only reduces fatty acyl-CoA substrates to the corresponding fatty aldehydes.[8] Likewise, the bacterial FACoAR from *Acinetobacter calcoaceticus* only reduces fatty acyl-CoA substrates to the corresponding fatty aldehyde.[9] A recent report identified a FACoAR from the cyanobacterium *Synechococcus elongatus* PCC7942 that catalyzes the reduction of a fatty acyl carrier protein (ACP) to a fatty aldehyde.[10] The enzyme was named a fatty acyl−acyl carrier protein reductase, as the enzyme had a lower K_m for fatty acyl-ACP versus fatty acyl-CoA,[10] which differentiates this from the

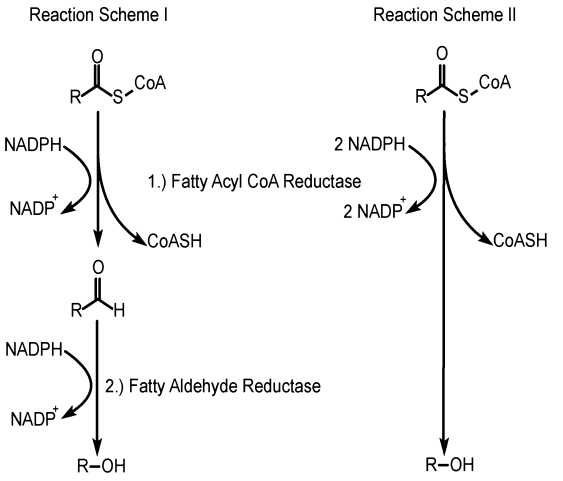


Figure 1. Proposed reaction schemes for fatty acyl-CoA reductase. Scheme I shows the two-step reduction mechanism proposed for known bacterial fatty acyl-CoA reductases. Scheme II shows the single-step reduction proposed for the fatty acyl CoA reductase enzymes of many higher eukaryotes.

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FACoAR from *A. calcoaceticus* that does not utilize fatty acyl-ACP.[9]

We recently characterized an enzyme from the marine bacterium *Marinobacter aquaeolei* VT8 that was found to reduce fatty aldehydes to fatty alcohols and had a higher specificity for long chain aldehydes than for shorter aldehydes.[11] We sought to also characterize the upstream enzyme in this pathway, a putative FACoAR, from the same organism. The FACoAR from *M. aquaeolei* VT8 is of particular interest because in addition to the C-terminus that shares similarity (74% similar and 53% identical between residues 370 and 660 of *M. aquaeolei* VT8 FACoAR) to the majority of the FACoAR from *A. calcoaceticus* (Figure 2) that was shown to reduce fatty acyl-CoA to the fatty aldehyde,[9] it also contains a separate N-terminal domain that is distinctive from the FACoAR of *A. calcoaceticus*. Here, we report the purification and characterization of the FACoAR from *M. aquaeolei* VT8 and show that this enzyme has broad substrate specificity and catalyzes the four-electron reduction of fatty acyl-CoA substrates to the corresponding fatty alcohols, making it unique among the characterized bacterial FACoAR enzymes.

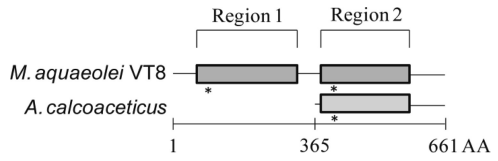

Figure 2. Domain arrangement of *M. aquaeolei* VT8 FACoAR compared to *A. calcoaceticus* FACoAR. Schematic domains are shown with the 661 amino acid *M. aquaeolei* VT8 and the 295 amino acid *A. calcoaceticus* sequence. Conserved regions are outlined with the C-terminal domain of the *M. aquaeolei* VT8 enzyme aligning with high similarity (53% identical and 74% similar) to the majority (residues 9 to 295) of the *A. calcoaceticus* enzyme. Denoted by asterisks are the conserved pyridine nucleotide binding regions found in each enzyme, which have the conserved sequence GXGX(1-2X)G.

MATERIALS AND METHODS

Reagents. 5′-Dithiobis(2-nitrobenzoic acid), also referred to as Ellman's reagent or DTNB, was purchased from TCI America (Portland, OR). Coenzymes (NADPH, NADH, $NADP^+$, and NAD^+), various fatty acyl-CoAs, fatty aldehydes and fatty alcohols, and all other reagents were purchased from Sigma-Aldrich, unless otherwise stated.

Cloning and Gene Expression. The protein sequence of the fatty acyl-CoA reductase (FACoAR) from *Acinetobacter calcoaceticus* (ZP_06058153.1)[9] was used to perform a BLAST search of the NCBI database for a corresponding gene in *Marinobacter aquaeolei* VT8. The search identified a gene (YP_959769.1) whose protein product contained 661 amino acids with ~50% identity (73% similarity) over a region that corresponded to about 280 residues of the C-terminus of the protein. The gene was cloned by PCR from purified genomic DNA isolated from *M. aquaeolei* VT8 using primers (GACGA-***GAATTC***AATTATTTCCTGACAGGCGGCACCGG) and (TCGAC***TCTAGA***CTCCAGTATATCCCCCGCATAATC) and the failsafe PCR kit (Epicenter, Madison, WI). The PCR product was ligated into the *Eco*RI and *Xba*I sites of a pUC derivative plasmid. The entire cloned insert was sequenced to confirm that no mistakes were introduced. The gene was then moved to a pMAL-c4x plasmid derivative (New England Biolabs, Ipswich, MA) containing an insert for incorporation of an 8X His-tag following the in-frame insertion after the *Xba*I site. This resulted in the final plasmid pPCRMALD8 that contains the FACoAR from *M. aquaeolei* VT8 with an N-terminal maltose binding protein (MBP) fusion and a C-terminal His-tag. This construct contains a Factor Xa cleavage site immediately following the MBP protein to facilitate removal of the maltose binding protein following purification. The plasmid was transformed into the *E. coli* TB1 strain (New England Biolabs, Ipswich, MA) for protein expression.

Protein was expressed by growing 1 L cultures in Luria–Bertani broth (LB) supplemented with 100 mg/L ampicillin from an 8 mL starter culture. The culture was grown with shaking at 37 °C until the culture reached an optical density of ~0.6 at 600 nm. Protein expression was induced by the addition of 50 mg/L of isopropyl-β-thiogalactopyranoside (IPTG), and the culture was grown for 3–4 h before harvesting by centrifugation. Collected cell pellets were frozen and stored at −80 °C.

Protein Purification. Cell pellets of ~5 g were resuspended in 30 mL of lysis buffer composed of 20 mM Tris-HCl pH 7.0, 50 mM NaCl, and 1 mM EDTA. The resuspended cells were placed in a 50 mL conical tube and placed in a water–ice mixture to keep the cells cold during lysis. The cells were passed through a French pressure cell three times at 1000 lb/in^2. Whole cell lysate was centrifuged at 10000*g* for 20 min to separate the cell debris from the soluble extract.

Soluble cell lysate was passed over an amylose column (P/N E8201L, New England Biolabs, Ipswich, MA) to bind the fusion protein and washed with 3 column volumes of lysis buffer, followed by a wash step with 2 column volumes of lysis buffer supplemented with 1.0 M NaCl to interrupt nonspecific binding. The column was then washed with 3 column volumes of equilibration buffer (20 mM Tris-HCl, pH 7.0, 50 mM NaCl), and the bound protein was eluted with 2 column volumes of equilibration buffer supplemented with 10 mM maltose. The relative protein concentrations of the collected fractions were determined by Nanodrop (Thermo Scientific, Wilmington, DE). Fractions containing significant amounts of protein were then pooled and added to a metal affinity column (P/N 17-0575-01, GE Healthcare, Upsalla, Sweden) charged with nickel. The metal affinity column was washed with 3 column volumes of equilibration buffer followed by a wash with 2 column volumes of equilibration buffer supplemented with 85 mM imidazole to disrupt nonspecific binding. The column was eluted with 2 column volumes of a 500 mM imidazole solution in equilibration buffer. Resulting fractions were analyzed on a 12% SDS-PAGE gel. Fractions containing a purified protein whose migration in the SDS-PAGE was consistent with a 116 kDa protein, according to the protein marker, were pooled and applied to a G25 Sephedex column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with equilibration buffer. Desalted fractions were flash frozen and stored in liquid nitrogen. Protein concentration was determined using the Pierce BCA protein concentration assay kit (Thermo Fisher Scientific, Rockford, IL).

Initial Activity Assays. Initial activity assays were conducted using thin layer chromatography (TLC) and a gas chromatography (GC) assay similar to that described previously.[11] To test activity, 0.3 mg of protein was added to a reaction vessel along with 200 μM palmitoleyl-CoA and 800 μM NADPH, NADH, $NADP^+$, or NAD^+ in reaction buffer containing 20 mM Tris-HCl, pH 7.0, and 50 mM NaCl.

Reactions were allowed to proceed for 1 h before extraction with 2 mL of hexane. The hexane water mixture was vortexed vigorously for 30 s before phase separation by centrifugation. The hexane phase was removed to a clean container, and the solvent was removed under a stream of argon gas. The resulting residue was resuspended in 100 μL of hexane and spotted on a TLC silica plate along with 5 μL each of palmitoleyl alcohol (10 mg/mL) and *cis*-11-hexadecenal (10 mg/mL) standards. Any unreacted palmitoleyl-CoA substrate would partition in the water phase and would not appear on the TLC plate. The TLC plate was developed in a 2:15:90 volumetric ratio of glacial acetic acid:ethyl ether:hexane. After development, visualization was performed in a sealed jar with iodine crystals for 10 min. The TLC results were verified by GC/MS analysis of the sample prepared in the same way. GC/MS results were compared to retention times and mass spectrum of known standards.

The GC/MS analysis was performed using a Shimadzu GCMS-QP2010S. A 1 μL sample was injected onto a Stabilwax-DA column (30 m × 0.25 mm i.d. with 0.25 μm film thickness (Restek, Belfonte, PA)) and run using a temperature program of 100 °C for 1 min, followed by a temperature gradient of 10 °C/min to 235 °C and held at the final temperature for 10 min. Helium gas was used as the carrier and flow was controlled by maintaining a constant velocity of 50 cm/min. Mass spectra obtained were compared to the National Institute of Standards and Technology (NIST) database 05 for peak identification

Continuous Spectrophotometric NADPH Assay. All assays were conducted in a total volume of 1 mL. A buffer containing 50 mM NaCl, 20 mM Tris-HCl pH 7.0, and 0.5 mg/mL bovine serium albumin was prepared along with a 1 mM stock of aldehyde dissolved in dimethyl sulfoxide (DMSO) or a 0.1 mM stock of the acyl-CoA and a 2.0 mg/mL stock of NADPH. All components including protein were degassed in sealed vials and placed under an argon atmosphere. NADPH was degassed as the solid prior to the addition of degassed buffer. Each assay was conducted by adding 75 μL of the NADPH stock, 58 μg of protein for acyl-CoA assays or 15 μg of protein for aldehyde assays, varying concentrations of aldehyde or acyl-CoA, and buffer to bring the final volume to 1 mL. Each sample was continuously monitored for the decrease of NADPH at 340 nm on a Varian 50 Bio UV−vis spectrophotometer (Walnut Creek, CA). Initial rates were calculated in Excel (Microsoft, Redmond, WA) using the linear initial rates of reaction obtained from the spectrophotometric assays by obtaining the slope from the best fit line and calculating nmol of NADPH oxidized per second. These initial rates were used to calculate the apparent K_m and V_{max} values using the Igor Pro software package (Wavemetrics, Lake Oswego, OR) fitting the initial rates to the Hill equation.[12] NADPH specific activity assays were conducted identically as described above using a fixed 60 μM concentration of the various aldehyde substrates or 5 μM of the various acyl-CoA substrates.

Continuous Spectrophotometric DTNB Assay. Buffers and solutions were prepared as described above for NADPH assays. Each assay was conducted by adding 75 μL of the 2 mg/mL NADPH stock solution in buffer, 58 μg of protein, and 10 μL of a 10 mg/mL solution of DTNB in DMSO, varying concentrations of acyl-CoA and buffer to bring the volume to 1 mL. Reduction of acyl-CoA substrate was monitored by following the increase of the 2-nitro-5-thiobenzoate (NTB^{2-}) dianion concentration at 412 nm. Initial rates were calculated in Excel (Microsoft, Redmond, WA) using the linear initial rates of reaction obtained from the spectrophotometric assays by obtaining the slope from the best fit line and calculating nmol of NTB^{2-} dianion formed per second using the extinction coefficient of the NTB^{2-} dianion of 14 150 M^{-1} cm^{-1}.[13,14] These initial rates were used to calculate the apparent K_m and V_{max} values using the Igor Pro (Wavemetrics, Lake Oswego, OR) software package fitting the initial rates to the Hill equation (eq 1) where v is the initial velocity, V_{max} is the maximum calculated velocity, $[S]^n$ is the concentration of substrate, n is the Hill coefficient, and $K_{0.5}^n$ is the approximation of K_m or the approximate substrate concentration at which half of V_{max} is obtained at a specific value of the Hill coefficient n. In the case of enzyme inhibition, a modified version of the Hill equation allowing for cooperative inhibition (eq 2) where all of the coefficients are defined as for the Hill equation and the additional $[i]^n$ is the concentration of inhibitor and n is the Hill coefficient. The K_i^n is the approximate concentration of inhibitor it takes to double the $K_{0.5}$ at a specific value of the Hill coefficient n.[12,15]

$$v = \frac{V_{max}[S]^n}{K_{0.5}^n + [S]^n} \quad (1)$$

$$v = \frac{V_{max}[S]^n}{K_{0.5}^n\left(1 + \frac{[i]^n}{K_i n}\right) + [S]^n} \quad (2)$$

pH Studies. Optimal pH was determined by assaying over a range of pH values from 5.5 to 9.0. A buffer composed of 50 mM MES, 50 mM MOPS, 50 mM TAPS, 150 mM NaCl, 0.5 mg/mL BSA was made, and the pH was adjusted by adding either NaOH or HCl. Assays were conducted using the NADPH continuous spectrophotometric assay described above.

Verification of Activity without MBP Tag. To determine activity of the enzyme with the maltose binding protein (MBP) removed, 500 μg of protein was digested with 10 units of Factor Xa (New England Biolabs, Ipswich, MA), according to the protocol provided by New England Biolabs. The resulting protein was used in a set of assays conducted according to the DTNB and NADPH protocols described above. The kinetic curves produced were compared to the established curves.

Determination of Quaternary Structure. 3 mg of purified protein was exchanged into a buffer containing 150 mM NaCl and 20 mM Tris-HCl pH 7.0. This protein was loaded onto a size exclusion column (High Load 2660 Superdex 200 GE Healthcare) equilibrated with a buffer containing 20 mM Tris-HCl pH 7.0 and 150 mM NaCl along with standards of known native molecular weight using the GE high molecular weight standard kit (General Electric Healthcare, Uppsala, Sweden) to determine the size of the resulting protein and run at 0.7 mL/min flow rate, and protein elution was monitored continuously at 280 nm.

RESULTS

The putative fatty acyl-CoA reductase (FACoAR) from *M. aquaeolei* VT8 was cloned as described in the Materials and Methods section following an approach similar to that used to clone and isolate active fatty aldehyde reductase (FALDR) from *M. aquaeolei* VT8.[11] The only variation in the approach taken here was the addition of a C-terminal 8X His-tag to the N-terminal maltose binding protein-FACoAR fusion protein.

This approach allowed a rapid two-step purification of the enzyme using amylose affinity and metal affinity chromatography (Figure 3). The migration of the protein on an SDS gel

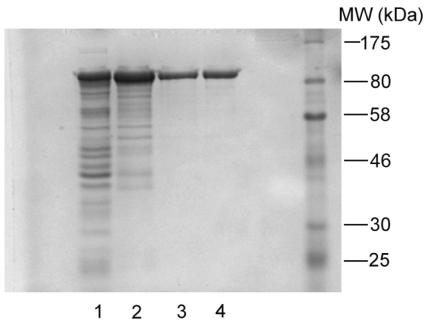

Figure 3. Purification of the FACoAR. Shown is an SDS-PAGE of the purification scheme of the FACoAR from *M. aquaeolei* VT8 expressed from *E. coli*. A protein of approximately 116 kDa is obtained after each affinity purification step. Lane 1 contains the soluble cell free lysate. Lane 2 contains the elution from the amylose affinity resin. Lane 3 contains the elution from metal affinity resin charged with nickel. Lane 4 contains the elution from the G25 sephadex column. Lane 6 contains the protein standards. Protein is >95% pure following the G25 sephadex column purification.

agreed well with the molecular mass of 116 kDa predicted from the amino acid sequence. Previous attempts to purify FACoAR proteins encountered solubility problems and resulted in inactive enzymes, similar to what was found for the FALDR.[9,11] By expressing the FACoAR from *M. aquaeolei* VT8 as a fusion with the maltose binding protein, the FACoAR was soluble and active.

An initial assessment of fatty acyl-CoA reduction activity was achieved by utilizing thin layer chromatography (TLC) to determine substrate and product identities and requirements for reductant. These initial assays established that reduction of a fatty acyl-CoA substrate by FACoAR required NADPH. When NADH was substituted for NADPH, no reaction product was detected. Further, while fatty acyl-CoAs were a substrate, no detectable reduction was found with free fatty acids. The TLC analysis revealed exclusively the fatty alcohol product, with no aldehyde detected (Figure 4).

The products of the FACoAR reduction of acyl-CoA were next analyzed by gas chromatography with flame ionization detection (GC/FID). Product identities were also confirmed by gas chromatography with detection by mass spectrometry (GC/MS). These assays verified that the only detectable product from palmitoleyl-CoA reduction by FACoAR was palmitoleyl alcohol. The requirement for NADPH agrees well with the findings by Reiser and Somerville for the FACoAR from *A. calcoaceticus*, which was also found to utilize NADPH as the reductant,[9] while eukaryotic FACoAR enzymes have been reported to use either NADPH for jojoba (*Simmondsia chinensis*) and honey bee (*Apis mellifera*) or NADH for the unicellular protist *Euglena gracilis*.[6,16,17]

To further examine the activity of the FACoAR, two real-time, spectrophotometric assays were developed. In one, the release of free CoA from reduction of an acyl-CoA was monitored by reaction of the CoA with Ellman's reagent (DTNB), which results in the production of the NTB^{2-} dianion that absorbs at 412 nm. In the second assay, the oxidation of NADPH was monitored at 340 nm. By using either the NADPH oxidation or the CoA release assay, the activity of the

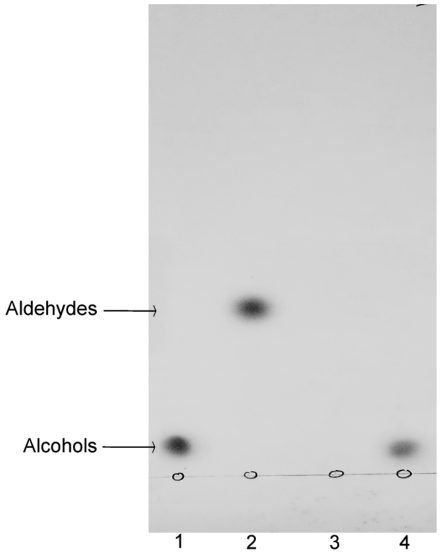

Figure 4. TLC plate of fatty acyl-CoA reductase products of reaction. Lane 1 contains 5 μL of a palmitoleyl alcohol (10 mg/mL in hexane) standard. Lane 2 contains 5 μL of a *cis*-11-hexadecenal (10 mg/mL in hexane) standard. Lane 3 contains heat inactivated FACoAR from *M. aquaeolei* VT8 incubated with palmitoleyl-CoA and NADPH and extracted as described in the Materials and Methods. Lane 4 is identical to lane 3, except that the FACoAR was not heat inactivated. Samples were allowed to react for 1 h with gentle shaking at room temperature before extracting with hexane and spotting on TLC. The solvent front is the top of the image. The drawn circles indicate the point at which the samples were blotted before developing the TLC plate.

enzyme could be followed with a range of substrates. Optimal activity was found for the enzyme at pH 7.0, with activity falling dramatically below pH 6.5 and above 9.0 (data not shown). Treatment of the FACoAR with the factor Xa protease to remove the maltose binding fusion protein did not result in a significant change in enzyme activity versus an uncleaved control (see Supporting Information). All further assays were done with the intact maltose binding protein−FACoAR fusion.

The fatty acyl-CoA substrates used in these experiments have relatively low critical micelle concentration (CMC) values.[18] The CMC values depend on the buffer ionic strength, temperature, and protein concentration present. For fatty acyl-CoA substrates, the generally accepted ranges for CMC values are between 10 and 40 μM. All specific activities were determined using fatty acyl-CoA concentrations below the published CMC values.[18−20] Experiments were conducted with higher concentrations of several fatty acyl-CoA substrates, resulting in the expected lower activity with higher substrate concentration (data not shown).

Figure 5 shows the specific activity for palmitoyl-CoA reduction versus the concentration of substrate, tracking either the formation of NTB^{2-} (the chromophore produced following reaction of DTNB with the free thiol of CoA) or the oxidation of NADPH. The data in both assays were best fit to a sigmoidal curve, indicating possible allosterism or cooperativity. Fitting the data to the Hill equation,[12] an apparent K_m of ~4 μM was obtained for the reduction of palmitoyl-CoA with a value of $n \sim 2.8$. The V_{max} was calculated to be ~115 nmol CoA released min^{-1} mg $protein^{-1}$ or ~197 nmol $NADP^+$ min^{-1} mg $protein^{-1}$, suggesting that the enzyme was catalyzing the four-electron reduction of the acyl-CoA to the corresponding alcohol.

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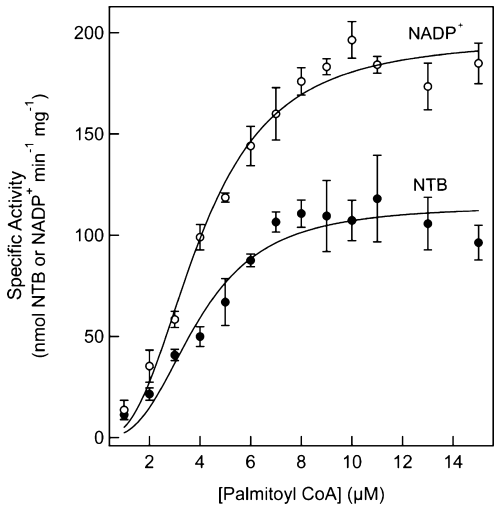

Figure 5. Kinetic parameters of fatty acyl-CoA reductase from *M. aquaeolei* VT8 indicating direct production of palmitoyl alcohol from palmitoyl CoA. The (●) indicate points for the DTNB assay measuring the release of free CoA. V_{max} of 115 ± 7 nmol NTB $^{-2}$ min $^{-1}$ mg of protein $^{-1}$, apparent K_m of 4 ± 0.3 μM, and a *n* of 2.8 ± 0.7. The (○) indicate points for the NADPH assay measuring the enzymatic utilization of NADPH. V_{max} of 197 ± 8 nmol $NADP^+$ min^{-1} mg of protein $^{-1}$, apparent K_m of 4.0 ± 0.2 μM, and *n* of 2.6 ± 0.3. All kinetic parameters were calculated using a triplicate data set with Igor Pro software shown with error bars representing the standard error of the mean (SEM) fit to eq 1 in the Materials and Methods.

In contrast to this FACoAR from *M. aquaeolei* VT8, Reiser et al. reported levels of activity for the *A. calcoaceticus* FACoAR of ~0.1 pmol min^{-1} μg $protein^{-1}$ (0.1 nmol min^{-1} mg $protein^{-1}$), although this was the activity reported for the unpurified cell extract, as purification was not achieved with their enzyme.[9]

The above data indicate that the *M. aquaeolei* VT8 FACoAR catalyzes the four-electron reduction of an acyl-CoA to the corresponding alcohol, and thus the reaction is expected to pass through the two-electron reduced aldehyde intermediate. In an attempt to trap a possible aldehyde intermediate, assays were performed in the presence of phenylhydrazine or hydrazine using an approach similar to that taken to isolate aldehyde intermediates from crude preparations of *E. gracillis* FACoAR.[17] Assays were conducted in the same manner as described in the Materials and Methods section for both NADPH and DTNB spectrophotometric assays, except that 5 mM phenylhydrazine or hydrazine was included in the buffer to potentially trap any free aldehyde. The results showed little difference between either the initial rate of NADPH oxidation or the total quantity of NADPH consumed between the sample with hydrazine and the control run without hydrazine (data not shown). To verify the reaction of hydrazine, samples were prepared with fatty aldehyde as substrate in the presence of 5 mM hydrazine or phenylhydrazine, and the reaction rate was measured by the oxidation of NADPH. Approximately 90% inhibition was measured in the samples containing hydrazine compared to the control without hydrazine. The lack of inhibition by hydrazine of the fatty acyl-CoA reduction to fatty alcohol indicates that a free fatty aldehyde intermediate is not accessible to hydrazine and likely is remaining bound to the enzyme and is therefore inaccessible to hydrazine reaction during the time frame of the assay.

The carbon length preference of fatty acyl-CoA substrates for the FACoAR from *M. aquaeolei* VT8 was also determined by examining the rates of substrate reduction for a series of fatty acyl-CoA molecules (Table 1). These results show palmitoyl-CoA as the best substrate. Good rates of reduction for longer (C20:4) and shorter (C8) fatty acyl-CoA groups were also found. These activities contrast with the results reported for the FACoAR from *A. calcoaceticus*, which showed significantly lower activity with substrates greater than C18 and no detectable activity with substrates smaller than C14.[9]

Table 1. Specific Activity toward Acyl-CoA Substrates

substrate	carbon chain length	specific activity (nmol NTB min^{-1} mg^{-1})[a]	% specific activity of palmitoyl-CoA reduction[b]
octanoyl-CoA	C8	15 ± 1	9
lauroyl-CoA	C12	34 ± 9	26
myristoyl-CoA	C14	34 ± 3	60
palmitoyl-CoA	C16	57 ± 6	100
palmitoleyl-CoA	C16:1	57 ± 3	99
stearoyl-CoA	C18	40 ± 2	69
oleyl-CoA	C18:1	44 ± 6	78
arachidonoyl-CoA	C20:4	49 ± 9	86

[a]Reactions were performed as described in the Materials and Methods section using 5 μM of the respective CoA and are reported as nmol of the NTB^{2-} anion produced per min per mg of fatty acyl-CoA reductase protein. [b]All values are reported as a percent of the specific activity for the reduction of palmitoyl-CoA by the fatty acyl-CoA reductase enzyme.

In addition to determining whether this FACoAR from *M. aquaeolei* VT8 could reduce fatty acyl-CoA substrates, the ability of the enzyme to reduce fatty aldehydes to the corresponding alcohol was determined. Such activity would be similar to the activity of the FALDR from this same species.[11] This activity was measured using the previously described spectrophotometric assay to track the disappearance of NADPH but utilized a range of fatty aldehydes as substrates (Table 2).

Table 2. Specific Activity toward Aldehyde Substrates

substrate	carbon chain length	specific activity (nmol $NADP^+$ min^{-1} mg^{-1})[a]	% specific activity of decanal reduction[b]
acetaldehyde	C2	9 ± 1	<1
propanal	C3	2 ± 1	<1
hexanal	C6	1200 ± 60	15
octanal	C8	3600 ± 670	45
decanal	C10	8000 ± 630	100
dodecanal	C12	7500 ± 570	93
cis-11-hexadecenal	C16:1	6300 ± 200	79
2-naphthaldehyde		460 ± 90	6

[a]Reactions were performed as described in the Materials and Methods section using 60 μM of the respective aldehyde and are reported as nmol of $NADP^+$ produced per min per mg of fatty acyl-CoA reductase protein. [b]All values are reported as a percent of the specific activity of decanal reduction by the fatty acyl-CoA reductase enzyme.

The best substrate was decanal (C10), with lower rates observed for shorter (to C6) and longer (to C16) aldehydes. Figure 6 shows the specific activity as a function of the concentration of *cis*-11-hexadecenal. Again, the products were analyzed by both TLC and GC/MS. A fit of these data to the Hill equation reveals an apparent K_m ~ 50 μM and V_{max} of ~8 μmol min^{-1} mg $protein^{-1}$, with cooperativity indicated by *n* ~ 1.9. The specific activity is significantly higher than the activity found for the

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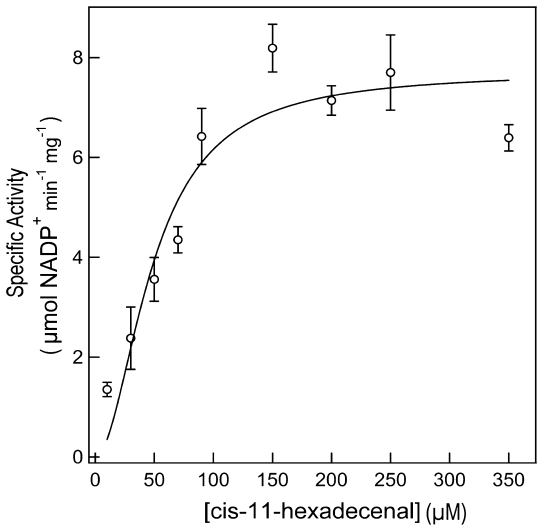

Figure 6. Kinetic parameters of fatty acyl-CoA reductase from *M. Aquaeolei* VT8 showing reactivity toward *cis*-11-hexadecenal. NADPH assay measuring the decrease in absorbance at 340 nm. V_{max} of 7.7 ± 0.6 μmol min^{-1} mg of protein $^{-1}$, apparent K_m of 48 ± 7 μM, and a n of 2 ± 0.8. Data shown with SEM. Calculated using Visual Enzymics and Igor pro Software fit to eq 1 in the Materials and Methods.

recently characterized FALDR enzyme from *M. aquaeolei* VT8[11] and more than 100-fold higher than the specific activity for reduction of palmitoleyl-CoA substrate (Table 1). Thus, the FACoAR described here can reduce either fatty acyl-CoA or fatty aldehyde substrates to the corresponding fatty alcohols.

For each of the fatty acyl-CoA substrates analyzed as part of this work (see Table 1), the quantity of NADPH oxidized during the reaction was found to be approximately twice the amount of NTB^{2-} (product of the reaction with DTNB and free CoA) produced. This result indicates that any fatty aldehyde formed from the reduction of fatty acyl-CoA is immediately further reduced to the fatty alcohol.

As is seen in Figures 5 and 6, the activities of the FACoAR from *M. aquaeolei* VT8 do not follow standard Michaelis–Menten kinetics. Instead, sigmoidal kinetics are observed that are best fit to the Hill equation. Cooperativity is observed with other enzymes that utilize acyl-CoA substrates and are active in the synthesis of lipid compounds. 3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductases from human, mammalian, and bacterial sources alike have been shown to demonstrate similar kinetic behavior.[21−23]

As a part of the characterization of the *M. aquaeolei* VT8 FACoAR, we probed the nature of the sigmoidal kinetics shown in Figures 5 and 6 by attempting to alleviate the sigmoidal response of activity as a function of substrate concentration by the addition of reducing agents and detergents, which have been shown to affect the cooperativity in the enzyme HMG-CoA reductase.[24] The use of detergents (isopropanol, Triton X-100) and reducing agents (β-mercaptoethanol) at varying concentrations failed to alleviate the sigmoidal character of the kinetics of the *M. aquaeolei* VT8 FACoAR and in each case inhibited activity (data not shown). This seems to indicate the potential of some inter- or intra-protein interaction that is necessary for activity to occur.

To further investigate this cooperativity, the protein was analyzed by size exclusion chromatography to determine the native size of the protein using a Superdex 200 size exclusion column (see Supporting Information). The protein traveled through the column as a single peak with a small amount of contaminating higher oligomers or aggregates traveling just ahead as a shoulder on the main peak. When compared to specific molecular weight standards, the primary peak ran with an apparent size ~4 times that of the predicted monomeric state, indicating a possible tetrameric form of the enzyme. When collected fractions were run on an SDS gel, all fractions were shown to contain only the single pure monomer of the FACoAR protein, yet only the fraction corresponding to the possible tetrameric state showed the highest levels of activity, while the higher contaminating oligomers were significantly lower in activity. This indicates that the enzyme likely requires a higher oligomeric state (tetramer) for activity but loses activity upon aggregation. These findings support the hypothesis that both the fatty acyl-CoA reduction and further fatty aldehyde reduction are performed by the same enzyme and are not the result of two different proteins, as the activity for both reactions remained constant through each step of the protein preparation.

To further probe the hypothesis that both fatty acyl-CoA reduction and fatty aldehyde reduction are performed by the same enzyme, and not the result of a contaminating protein, inhibition experiments were devised. Since the specific activity for fatty acyl-CoA is lower (100-fold lower) than the specific activity for aldehyde reduction, the addition of fatty acyl-CoA would be expected to inhibit fatty aldehyde reduction in a concentration-dependent manner. Further, if both substrates are utilizing the same enzyme (or potentially even the same active site), then the substrates would be expected to act as reversible inhibitors of one another and should shift the apparent K_m, but not change the V_{max}. In fact, it is observed that the addition of increasing concentrations of palmitoyl-CoA (1, 2, and 4 μM) significantly lowered the enzymatic activity for aldehyde reduction with an increase in the apparent K_m (Figure 7) and

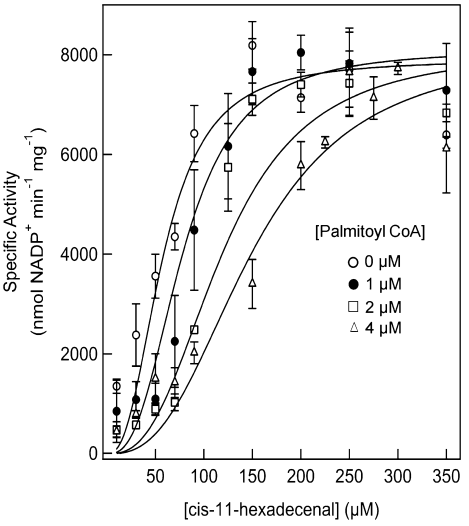

Figure 7. Palmitoyl CoA inhibition of fatty aldehyde reduction. The reduction of the fatty aldehyde *cis*-11-hexadecenal is inhibited in a competitive manner with an apparent K_i of 1.9 ± 0.26 μM palmitoyl-CoA. The (○) denote assays performed with 0 μM palmitoyl-CoA, (●) denote assays performed with 1 μM palmitoyl-CoA, (□) denote assays performed with 2 μM palmitoyl-CoA, and (△) denote assays performed with 4 μM palmitoyl-CoA. Specific activities are plotted in units of nmol NADP$^+$ min^{-1} mg protein^{-1}. All assays were conducted in triplicate and are shown with error bars indicating SEM fit to eq 1 in the Materials and Methods section. The apparent K_i was determined by fitting all four data sets to eq 2 in the Materials and Methods.

was estimated based on a fit using eq 2 to have a K_i of ~2 μM. This inhibition could be overcome with the addition of higher

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concentrations of aldehyde substrate, suggesting competition within the same enzyme by the palmitoly-CoA.

The inverse inhibition experiment tracking the effect of added fatty aldehyde on fatty acyl-CoA reduction using the DTNB assay (which is specific for tracking CoA release) was also of interest to determine if fatty aldehyde addition would alter the apparent K_m for the fatty acyl-CoA. However, concentrations of fatty aldehyde required (greater than 150 μM to saturate the enzyme) for this experiment resulted in elevated background interference of the aldehyde with the DTNB reagent, making it difficult to have sufficient confidence in the results obtained.

DISCUSSION

The fatty acyl-CoA reductase (FACoAR) from *A. calcoaceticus* (ZP_06058153.1) was the first FACoAR described in a bacterium[9] and was utilized as an initial template to search for a similar enzyme in *Marinobacter aquaeolei* VT8. An enzyme from *M. aquaeolei* VT8 was found (YP_959769) with amino acid sequence similarity to the FACoAR from *A. calcoaceticus*, although the *M. aquaeolei* VT8 enzyme appears to have an additional N-terminal domain. The first domain on the N-terminal end of this enzyme shares a very slight sequence similarity (41% similar and 22% identical over the region between residues 4 and 145) with the fatty aldehyde reductase (FALDR) previously characterized from *M. aquaeolei* VT8,[11] while the second domain on the C-terminal end (beginning at ~375 residues and proceeding to the end of the protein) aligns well (74% similar and 53% identical) with the FACoAR from *A. calcoaceticus* (Figure 2). These similarities are consistent with the *M. aquaeolei* VT8 FACoAR enzyme catalyzing the four-electron reduction of a fatty acyl-CoA substrate to the corresponding fatty alcohol as reported here, making this bacterial enzyme more similar in reactivity to the FACoAR described from the plant jojoba.[6] Although the *M. aquaeolei* VT8 FACoAR does not align well with known acyl-CoA reductases from plants, a high sequence similarity for the entire sequence to a range of proteins from many other bacteria was noted, including enzymes found in lipid accumulating bacteria such as *Rhodococcus*,[25,26] *Alcanivorax*,[27] and several strains of *Mycobacterium*.[28]

The FACoAR from *M. aquaeolei* VT8 was found to catalyze the NADPH-dependent reduction of fatty acyl-CoA substrates ranging from 8 to 20 carbons in length (both saturated and unsaturated) as well as fatty aldehyde substrates to the corresponding fatty alcohol. The *M. aquaeolei* VT8 FACoAR activity is significantly higher than what was reported for FACoAR from *A. calcoaceticus*.[9] The *M. aquaeolei* VT8 FACoAR also exhibited an independent fatty aldehyde reductase activity, which was not reported for the *A. calcoaceticus* FACoAR enzyme. The *M. aquaeolei* VT8 FACoAR only acted on fatty acyl groups bound to CoA and did not react with free fatty acids directly.

The FALDR from *M. aquaeolei* VT8 characterized previously in our laboratory[11] shares a minimal sequence similarity with the CER4 protein from *Arabidopsis*[7] and also shares a very minimal similarity with the N-terminal domain for the FACoAR described here. The FALDR enzyme was previously tested against a range of fatty aldehydes and for the same substrate as shown in Figure 6 (*cis*-11-hexadecenal) was found to have a K_m of 177 μM and a V_{max} of ~60 nmol min^{-1} mg protein^{-1}.[11] From these results, the FACoAR described here appears to have a lower apparent K_m and a much higher V_{max} overall than was reported for the *M. aquaeolei* VT8 FALDR for fatty aldehyde reduction. To verify that the FALDR previously isolated from *M. aquaeolei* VT8[11] did not have activity toward fatty acyl-CoA substrates, it was subjected to the same DTNB and NADPH assays described here for the FACoAR. Activity was confirmed for fatty aldehyde reduction as previously reported, but no activity was detected (<1.0 nmol min^{-1} mg^{-1}) for fatty acyl-CoA reduction, indicating a clear difference for the substrate profiles of these two enzymes.

M. aquaeolei VT8 is a member of the hydrocarbonoclastic bacteria, a group of cosmopolitan oil-degrading marine bacteria that have a narrow substrate range that includes alkanes, polycyclic aromatic hydrocarbons, and small organic acids.[29] Hydrocarbon degradation would require a range of enzymes capable of oxidizing alkanes to serve as a source of energy, and thus the FACoAR could presumably be involved in degradation pathways in addition to production of fatty alcohol. To test whether FACoAR might play a role in oxidative reactions, the reversibility of this enzyme was tested using $NADP^+$ and various substrates. No activity was detectable following the reduction of $NADP^+$ at 340 nm (<1.0 nmol min^{-1} mg protein^{-1}) in the presence of fatty alcohols, fatty aldehydes, or combinations of each with free CoA, though this may simply be the result of an equilibrium that strongly favors the alcohol and $NADP^+$ products.

A possible mechanism of action for the *M. aquaeolei* VT8 FACoAR can be considered by comparison to mechanisms proposed for related enzymes. Although there are no reports of enzymes fully kinetically characterized with high sequence identity to this enzyme, the HMG-CoA reductase provides a model for comparison. HMG-CoA reductase is an important enzyme in the cholesterol synthase pathway that catalyzes the four-electron reduction of HMG-CoA to mevalonate via mevaldehyde as an intermediate. In HMG-CoA reductase, the two reduction steps are believed to occur at one active site where the CoA substrate is reduced to the alcohol completely, with the mevaldehyde intermediate not leaving the active site. Figure 8 shows a possible reaction scheme for the FACoAR catalyzed reduction of a fatty acyl-CoA substrate that parallels the mechanism for the HMG-CoA reductase and is supported by the experimental data presented herein. The lack of an effect

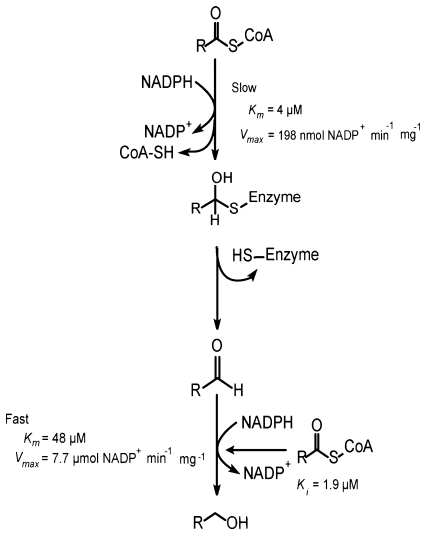

Figure 8. Proposed reaction mechanisms for fatty acyl-CoA reductase. This figure shows the reduction of the fatty acyl-CoA occurring within the same active site in a two-step reduction. The inhibition of aldehyde reduction by fatty acyl-CoA is shown. The reaction could proceed through an aldehyde intermediate.

of either hydrazine or phenylhydrazine on FACoAR rates of fatty acyl-CoA reduction supports a model where the intermediate aldehyde does not diffuse away from the enzyme before being further reduced to the alcohol. As each of the domains of the *M. aquaeolei* VT8 FACoAR contain a conserved pyridine nucleotide binding region, there is a potential for two separate active sites in the enzyme, though further experiments involving truncations will be required to adequately test this possibility.

The V_{max} for fatty aldehyde reduction by the FACoAR is significantly higher than the V_{max} obtained for fatty acyl-CoA substrate reduction in this FACoAR, although the apparent K_m for fatty acyl-CoA was somewhat lower (~4 μM for palmitoyl-CoA versus ~50 μM for *cis*-11-hexadecenal, Figures 5 and 6). This higher rate of reduction for the aldehyde could be a key factor in minimizing the loss of a potential intermediate aldehyde that could be toxic to the cell. Inhibition experiments using combinations of both fatty aldehyde and fatty acyl-CoA revealed that small concentrations of fatty acyl-CoA inhibited fatty aldehyde reduction in a concentration-dependent manner that could be overcome with higher concentrations of fatty aldehyde (Figure 7). These data, along with a consistent ratio of activities for both fatty aldehyde reduction and fatty acyl-CoA reduction in all steps of the purification including the size exclusion chromatography step, support the hypothesis that both activities are the result of the same enzyme and support a likelihood that both reactions occur either at the same active site or at active sites that are strongly cooperative with one another. The second hypothesis is supported by the presence of two conserved pyridine nucleotide binding regions within the protein and may indicate that even with two active sites, only one of the active sites is able to catalyze the related reaction at a time.

The sigmoidal character of the rate versus substrate concentration (Figures 5 and 6) can be explained by protein−protein interactions that might occur in a multimeric form of the protein, such as the homotetramer that is indicated from our results. The interacting proteins would be cooperating to allow enzymatic function much like what is seen with HMG-CoA reductase.[24] For HMG-CoA reductases, cleavage of sections of protein with freeze/sheer solubilization led to elimination of the sigmoidal kinetics, indicating a loss of cooperativity.[24] To test whether portions of the N- or C-terminus of the protein are responsible for the exhibited cooperativity and necessary for catalysis, truncated versions of *M. aquaeolei* VT8 FACoAR could be constructed as part of future work.

In this report, we have described a novel bacterial enzyme from *M. aquaeolei* VT8 that catalyzes the four-electron reduction of fatty acyl-CoA substrates to the corresponding fatty alcohol, in contrast to other reports for bacterial FACoAR enzymes that only reduce fatty acyl-CoA by two electrons to the fatty aldehyde.[9] The C-terminal domain of the FACoAR from *M. aquaeolei* VT8 shares sequence similarity with the FACoAR from *A. calcoaceticus*, while the unique N-terminus domain appears to have little homology to other known FACoAR enzymes. Further, the substrate specificity for the *M. aquaeolei* VT8 FACoAR is broader than the relatively narrow specificity reported for the vast majority of other FACoAR enzymes previously characterized.[6,9,16,30] Homologues to the *M. aquaeolei* VT8 FACoAR are found in a variety of other bacteria, including those that are known to accumulate wax esters, indicating that this enzyme may constitute an additional class of bacterial FACoAR enzymes in contrast to those only sharing similarity with the single domain FACoAR from *A. calcoaceticus*.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Factor Xa protease cleavage with activity comparisons and native protein size determination results. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

CoA, coenzyme A; FACoAR, fatty acyl-CoA reductase; NADPH, reduced nicotinamide adenine dinucleotide phosphate; $NADP^+$, oxidized nicotinamide adenine dinucleotide phosphate; DTNB, 5′-dithiobis(2-nitrobenzoic acid); NTB, 2-nitro-5-thiobenzoate; CMC, critical micelle concentration; FALDR, fatty aldehyde reductase.

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