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The thiamine-dependent enzyme of the vitamin K biosynthesis catalyzes reductive C–N bond ligation between nitroarenes and α-ketoacids

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The thiamine-dependent enzyme (1*R*, 2*S*, 5*S*, 6*S*)-2-succinyl-5-enolpyruvyl-6-hydroxyl-3-cyclohexene-1-carboxylate (SEPHCHC) synthase, also known as MenD, catalyzes a Stetter-like reaction in the biosynthesis of vitamin K. It is found to catalyze a novel reductive C–N bond ligation reaction between nitroarenes and α-ketoacids to form *N*-hydroxamates. This reaction likely proceeds through an enzyme-mediated, slow two-electron reduction of the nitroalkanes to form a nitroso intermediate, which serves as the electrophilic acceptor of the ketoacid-derived acyl anion. The involvement of the nitroso intermediate is supported by the fact that similar *N*-hydroxamates are readily formed at a much higher rate when nitroso compounds replace the nitro substrates in the chemoenzymatic reactions. These results demonstrate that the thiamine-dependent enzyme is able to catalyze novel, nonnative reactions that may find new chemoenzymatic applications.

chemoenzymatic synthesis, *N*-hydroxamates, thiamine-dependent enzymes, SEPHCHC synthase, vitamin K biosynthesis

1 Introduction

Thiamine diphosphate (ThDP)-dependent enzymes typically convert an electron-deficient carbonyl substrate into a nucleophile in their catalysis. An important step in their catalysis is activation of the ThDP coenzyme through deprotonation of C2-proton of the thiazolium ring. The resulting ylide then attacks an α-ketoacid substrate to generate an enamine carbanion intermediate or acyl anion after decarboxylation (Figure 1) [1–3]. Subsequently, the electron-rich intermediate undergoes rearrangement, oxidation, or nucleophilic addition to electrophilic acceptors to form new carbon-carbon bonds. Over the last several decades, a large number of ThDP-dependent enzymes have been discovered to form a large enzyme superfamily catalyzing a broad range of reactions in microorganisms, plants, and mammals in diverse biological processes [4].

The carbon-carbon bond forming ThDP-dependent enzymes are attractive targets for biotechnological exploitation as versatile biocatalysts for chemoenzymatic synthesis [5, 6]. Pyruvate decarboxylase (PDC) and benzoylformate decarboxylase (BFD) are two successful examples of such efforts, although both enzymes do not catalyze carbon-carbon bond formation in their physiological activities. Remarkably, they tolerate a wide range of structural variations in their substrates, allowing access to a large number of enantiopure products. For decades, yeast PDC has been used to prepare the (*R*)-phenylacetyl carbinol precursor by fermentation for industrial production of (1*R*, 2*S*)-ephedrine [7, 8], a versatile chiral synthon in organic synthesis [9, 10] and an agonist of noradrenaline receptors with clinical applications [11]. In addition, transketolase is another successful biocatalyst in chemoenzymatic synthesis [12]. It

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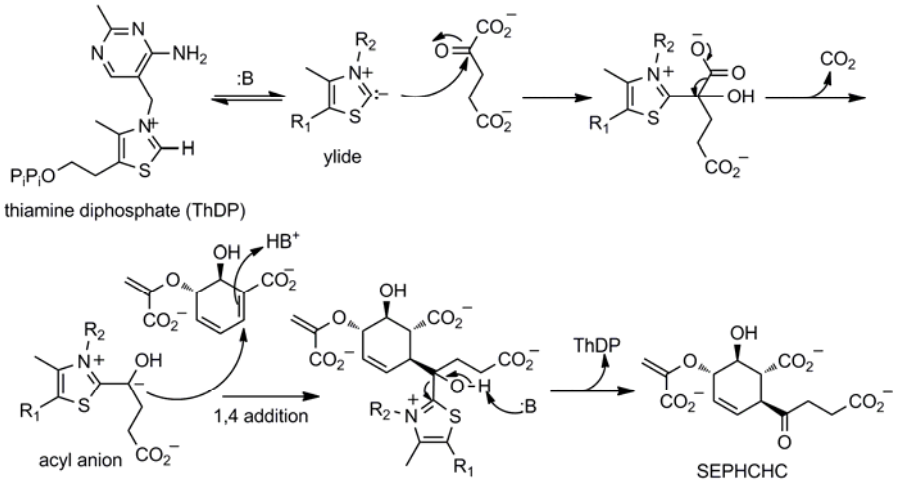


Figure 1 The proposed catalytic mechanism of SEPHCHC synthase. SEPHCHC: (1*R*, 2*S*, 5*S*, 6*S*)-2-succinyl-5-enolpyruvyl-6-hydroxyl-3-cyclohexene-1-carboxylate; P_i: phosphate group.

transfers a glycoaldehyde unit from either a donor ketose or hydroxypyruvate to an α-hydroxyaldehyde acceptor and has been used to synthesize a number of bioactive molecules [13–16]. It has also been suggested for use as an alternative to the versatile aldolase for synthesis of a number of natural products [17].

Recently, a new ThDP-dependent enzyme called MenD or (1*R*,2*S*,5*S*,6*S*)-2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate (SEPHCHC) synthase has been characterized in the biosynthesis of menaquinone (vitamin K2) [18, 19]. Different from all other carbon-carbon bond forming ThDP-dependent enzymes that use carbonyl compounds as the electrophilic acceptor, this enzyme uses isochorismate, an α,β-unsaturated carboxylate, as the electrophilic acceptor of the acyl anion generated from 2-oxo-glutarate. Structural and mechanistic studies have confirmed that the enzyme contains a conserved glutamate to activate the ThDP cofactor as a thiazolium ylide, similar to other ThDP-dependent enzymes [20, 21]. Its catalytic mechanism is proposed as shown in Figure 1, which is similar to that of other ThDP-dependent enzymes except for the nucleophilic 1,4-Michael addition. The finding of this enzyme has provided new opportunity for exploitation of the ThDP-enzymes in chemoenzymatic syntheses. Indeed, the MenD orthologue from *E. coli* has been shown to use unnatural carbonyl compounds as the electrophilic acceptor of the acyl anion to form stereospecific products [22].

In attempt to use MenD for chemoenzymatic synthesis of γ-oxo carboxylic acids, the enzyme was tested for its activity towards unnatural α, β-unsaturated carboxylates such as 4-nitrocinnamic acid. Unfortunately, the enzyme was found to exhibit a high substrate specificity and no expected 1, 4-addition product was formed. Quite unexpectedly, *N*-hydroxamates were formed from nitro-containing compounds and α-ketoacids through a reductive C–N bond ligation likely involving a nitro intermediate. The involvement of the nitroso intermediate is supported by the formation of similar *N*-hydroxamates from nitroso compounds and α-ketoacids. The finding of this C–N bond ligation reaction has provided new opportunities to exploit the ThDP-dependent enzymes in chemoenzymatic synthesis.

2 Materials and methods

2.1 General information

2-Oxo-glutarate, thiamine diphosphate (ThDP), 4-nitrocinnamic acid (97%, predominantly in *trans*-configuration), isopropyl β-D-thiogalactopyranoside (IPTG), buffers, and salts were purchased from Sigma-Aldrich. Other chemicals and biochemicals were obtained from commercial sources with the highest possible quality and used without further treatment, unless stated otherwise. Protein chromatography was performed on a Bio-Rad Bio Logic HR Workstation in a refrigerated chamber. HPLC analysis and purification were carried out using a Waters 600E system with Model 2487 dual λ absorbance detector. UV-vis absorbance was measured using a Perkin-Elmer Lambda 900 UV/vis/NIR spectrometer. The molecular weight of the products from enzymatic reactions was determined by high resolution electron-spray ionization (ESI) mass spectroscopy. Nuclear magnetic resonance (NMR) spectra were taken on 400 MHz Bruker spectrometers.

2.2 Expression, purification and activity test of *bs*MenD

The MenD orthologue from *Bacillus subtilis* (*bs*MenD) was expressed using a recombinant plasmid containing the target gene in a modified version of the Novagen pET15b vector, in which the thrombin cleavage site is replaced by a Tobacco Etch Virus (TEV) protease site. The recombinant plas-

mid was a generous gift from professor William N. Hunter of the University of Dundee [21]. To obtain the enzyme, the plasmid was introduced into the host cell *E. coli* BL21 (DE3) and the gene product was overexpressed in Luria broth containing 0.5 mM IPTG at 16 °C for 18 h. The expressed enzyme was then purified from the crude extract to greater than 95% purity by a 5.0 mL HiTrap chelating HP column (GE Healthcare). The purified protein was quantified by a Coomassie Blue protein assay kit (Pierce) and stored in phosphate buffered saline (pH 7.4) containing 10% glycerol at −20 °C until use.

For determination of the *bs*MenD activity, EntC obtained in previous studies [23, 24] was used to prepare isochorismate from chorismate, which was isolated from an engineered bacterial strain using a reported procedure [25]. The enzyme activity was either determined by monitoring the decrease of absorbance at 278 nm due to the consumption of isochorismate [18] or by monitoring the absorbance increase at 293 nm in an assay using the (1*R*,6*R*)-2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC) synthase MenH [26, 27] as the coupling enzyme. The kinetic parameters V_{max} and K_M were determined in the presence of saturating concentration of the cofactors and one of the substrates. All kinetic assays were carried out in 50 mM Tris buffer (pH 7.8) containing 50 mM NaCl and 20 mM $MgCl_2$ at 23 °C.

2.3 *bs*MenD activity with nonphysiological substrates

Taking 4-nitrocinnamic acid as the substitute of isochorismate for an example, the reaction rate was monitored in real time by full wavelength UV scanning. UV-vis spectra were taken using a Perkin-Elmer Lambda 900 UV/vis/ NIR spectrometer. A typical reaction mixture contained 200 μM 4-nitrocinnamic acid, 2 mM 2-*oxo*-glutarate, 200 μM ThDP in 50 mM Tris buffer (pH 7.8) supplemented with 50 mM NaCl and 20 mM $MgCl_2$ at room temperature. The reaction was initiated by adding the enzyme to a varied concentration. Since 2-*oxo*-glutarate was continuously consumed to form 2-hydroxy carbonyl products as shown previously [22], fresh substrate was added to an equivalent final concentration of 2 mM every 2 h during the reaction. This replenishment of 2-*oxo*-glutarate was also performed in activity tests using other electrophilic acceptors as substitute of the isochorismate substrate. In tests where the 2-*oxo*-glutarate substrate was also replaced, concentration of the substituting ketoacid was not fixed but varied in a range from 200 μM to 10 mM while keeping other reaction components unchanged.

In addition to the spectroscopic monitoring, the reaction products were also monitored and quantified by HPLC. To do this, the reaction was stopped by removing the enzyme through ultrafiltration and HPLC analysis was performed on a Waters 600E system with Model 2487 dual λ absorbance detector set at the characteristic ultraviolet absorption wavelength of the reactant or the product. The reaction mixture was analyzed on an Nova-Pak C18 reverse-phase column (4 μm particle size, 3.9 × 150 mm) using a linear gradient from 100% solution A to 100% solution B over 60 min at a flow rate of 1 mL/min, where solution A was 0.1% formic acid water solution and solution B was 100% acetonitrile. Control reactions without enzyme were treated in parallel and analyzed under the same conditions. To quantify the product formed in the enzymic reactions, 2-nitrobenzoic acid was added into the mixture to a final concentration of 100 μM and was used as an internal standard in the HPLC analyses. Integration areas of the elution peaks of the chromogenic substrate (such as 4-nitrocinnamic acid) and the internal control with known concentrations were calibrated to determine conversion rate of the substrate at different reaction times.

2.4 Preparation of *N*-[4-(2′-carboxy-(*E*)-vinyl)phenyl] succinyl hydroxamate (1) and other turnover products

Enzymatic preparation of product **1** was performed in 5 mL of 50 mM Tris buffer (pH 7.8) containing 2 mM 4-nitrocinnamic acid, 4 mM 2-*oxo*-glutarate, 10 mM $MgCl_2$, 200 μM ThDP and 20 μM MenD. 2-*oxo*-Glutarate was replenished to a final concentration of 4 mM every 6 h during the reaction, assuming that the previously added substrate was completely consumed. The reaction mixture was incubated at room temperature for 2 d (> 60% consumption of 4-nitrocinnamic acid) and quenched by addition of HCl to pH ~1. The products were extracted with ethyl acetate (4 × 5 mL). The organic extracts were combined and the organic solvent was removed under reduced pressure. The residue was re-suspended in 1 mL mixture of water and acetonitrile (1:1) and subjected to purification by a semi-preparative HPLC column. The product fractions were pooled, concentrated under reduced pressure to remove acetonitrile, and then lyophilized to obtain the product with 60% yield (4-nitrocinnamic acid as the limiting agent). Other turnover products of the nonphysiological reactions of *bs*MenD were similarly prepared.

3 Results and discussion

3.1 Reductive C–N bond ligation between 4-nitrocinnamic acid and 2-oxo-glutarate

*bs*MenD was overexpressed to a very high level in *E. coli* and a yield of 100 mg protein per 1 liter shake-flask culture was readily attainable. The purified enzyme was homogenous after metal chelating column chromatography and exhibited an activity consistent with results from previous characterization of the enzyme [21]. To explore the substrate tolerance of the enzyme, cinnamic acid and its derivatives were used as substitute for the isochorismate substrate in the *bs*MenD-catalyzed reaction, which was monitored by

ultraviolet spectroscopy and HPLC analysis. Interestingly, when 4-nitrocinnamic acid was used in the reaction, the electrophilic compound was obviously modified. As shown in Figure 2(a), the characteristic absorption of 4-nitrocinnamic acid at 312 nm gradually decreased over time while a new absorption peak at 270 nm emerged and its intensity increased as the reaction proceeded. Reverse-phase HPLC analysis of the enzymic reaction mixture confirmed that 4-nitrocinnamic acid was indeed transformed into a new product with a higher polarity (Figure 2(b)) and the conversion was complete (> 99%) after 48 hours of incubation at room temperature. However, no spectroscopic or HPLC changes were observed when cinnamic acid, 4-bromocinnamic acid, or 4-methyl-cinnamic acid was used in the enzymic reaction (data not shown). In all these reactions, 2-*oxo*-glutarate undergoes benzoin-like condensation as shown previously [22], but this reaction is not detectable by the employed method due to the lack of ultraviolet absorption for the ketoacid substrate and the condensation product. This ketoacid substrate was replenished as indicated in the methods and materials to compensate for the loss of the substrate during the reaction.

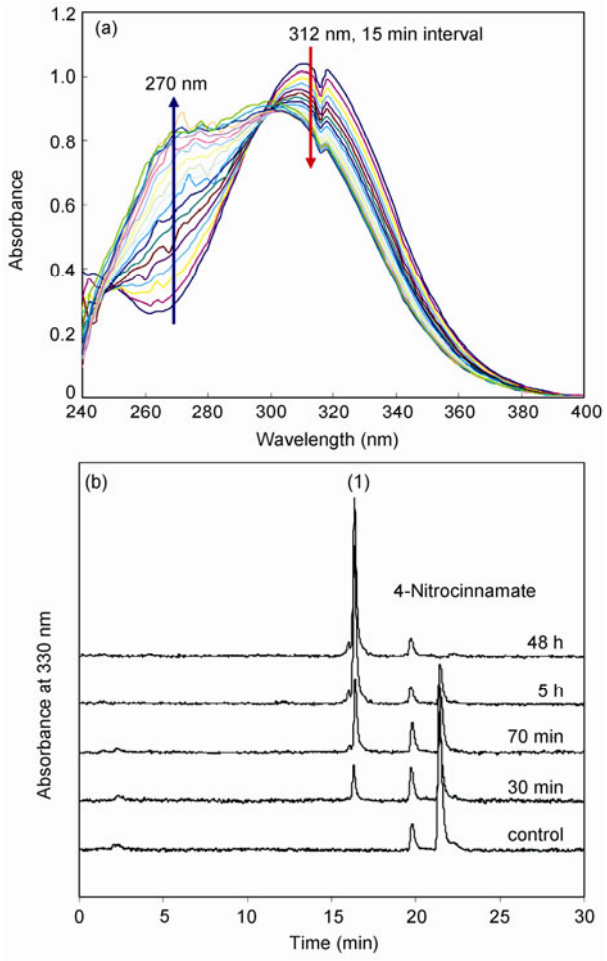


Figure 2 The *bs*MenD-catalyzed reaction of 4-nitrocinnamic acid and 2-oxo-glutarate. (a) UV-vis spectroscopic monitoring of the reaction. Spectra were taken every 15 min; (b) HPLC analysis of the reaction product at different time points. The reaction was carried out at 25 °C in a solution containing 65 μM 4-nitrocinnamic acid, 2 mM 2-ketoglutarate, 100 μM ThDP and 7.4 μM *bs*MenD in 50 mM Tris buffer (pH 7.8) supplemented with 50 mM NaCl and 20 mM $MgSO_4$. Control reaction did not contain the enzyme.

The turnover product from the *bs*MenD-catalyzed reaction between 4-nitrocinnamic acid and 2-oxo-glutarate was purified by HPLC from the reaction mixture for structural determination. ^{1}H NMR spectroscopy reveals a C=C double bond in the product, as indicated by two olefinic protons in *trans*-configuration at 7.69 (d, J = 16.0 Hz, 1H) and 6.52 (d, J = 16.0 Hz, 1H), demonstrating that the expected 1, 4-Michael addition of the acyl anion to the electrophilic 4-nitrocinnamic acid does not occur in the reaction. The product has a molecular formula of $C_{13}H_{13}NO_6$, which has a calculated mass of 279.0743, consistent with the major molecular ions $[M + H]^+$ at m/z = 280.0831 and $[M + Na]^+$ at m/z = 302.0675 in the mass spectrometry. Using COSY, HMBC and HMQC techniques, all ^{1}H NMR signals of the product are assigned as shown in Table 1. These results show that the product is *N*-[4-(2′-carboxy-(*E*)-vinyl)phenyl] succinyl hydroxamate (**1**), which is obviously formed from reduction and nucelophilic addition at the nitro group of 4-nitrocinnamic acid. Since no reaction was observed for cinnamic acid or its other derivatives without a nitro group, the nitro group may be the only structural element required for the observed reaction.

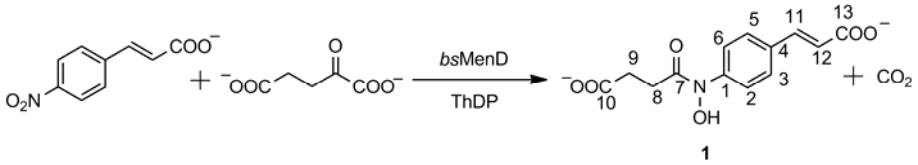
3.2 Reductive C–N bond ligation between other nitro compounds and 2-*oxo*-glutarate

Inspired by the result for 4-nitrocinnamic acid, several other nitro compounds were subjected to *bs*MenD-mediated reaction with 2-*oxo*-glutarate. As shown in Table 2, 3-nitrobenzoate, 4-nitrobenzoate and nitrobenzene indeed undergo reductive coupling reaction with 2-*oxo*-glutarate to form corresponding hydroxamates, whereas no reaction occurs for other nitro compounds. The lower conversion rate for nitrobenzene is probably due to its low aqueous solubility, which may also underlie the lack of reactivity for nitroethane. Interestingly, all the inactive nitro compounds contain an electron-donating group to increase the electron density of the nitro group, thereby decreasing its oxidative potential. These results suggest that a high oxidative potential is needed for the reductive coupling reaction.

Characterization data for *N*-(4-carboxyphenyl) succinyl hydroxamate (**2**) from 4-nitrobenzoic acid: $C_{11}H_{11}NO_6$, calcd. 253.0586; found 254.0692 ($[M + H]^+$) and 276.0516 ($[M + Na]^+$). δ_H (400 MHz, CD_3OD): 3.06 (2H, t, J = 6.40 Hz,), 2.71 (2H, t, J = 6.40 Hz), 7.87 (2H, d, J = 8.80 Hz), and 8.07 (2H, d, J = 8.80 Hz).

Characterization data for *N*-(3-carboxyphenyl) succinyl hydroxamate (**3**) from 3-nitrobenzoic acid: $C_{10}H_{11}NO_6$, calcd. 253.0586; found 254.0693 ($[M + H]^+$) and 276.0513 ($[M + Na]^+$). δ_H (400 MHz, CD_3OD): 3.02 (2H, br, s), 2.70

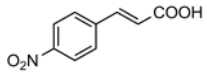
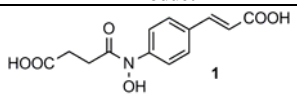
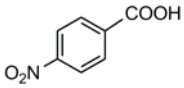
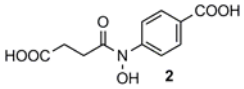
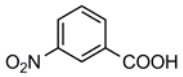
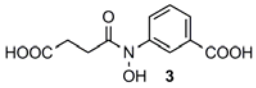
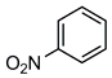
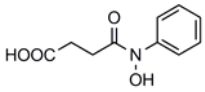
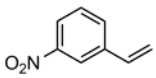
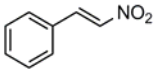
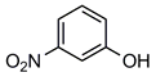
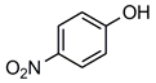
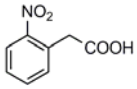
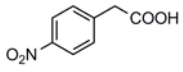
Table 1 Assignment of the NMR signals of the product *N*-[4-(2′-carboxy-(*E*)-vinyl)phenyl]-succinyl hydroxamate (1)



1

Position	δ ^{1}H (ppm)	δ ^{13}C (ppm)	COSY	HSQC	HMBC
C1	no proton				
C2	7.79, d, 2H, *J* = 4.4 Hz	118.9	7.66	118.9	
C3	7.66, d, 2H, *J* = 4.4 Hz	127.4	7.79	127.4	127.9, 143.4
C4	no proton	127.9			
C5	7.66, d, 2H, *J* = 4.4 Hz	127.4	7.66	127.4	127.9, 143.4
C6	7.79, d, 2H, *J* = 4.4 Hz	118.9	7.79	118.9	
C7	no proton				
C8	3.03, t, 2H	28.2	2.72	28.2	
C9	2.72, t, 2H	27.4	3.03	27.4	173.9, 28.2
C10	No proton	173.9			
C11	7.69, d, 1H, *J* = 16 Hz	143.4	6.52	143.4	127.9, 169.9
C12	6.52, d, 1H, *J* = 16 Hz	117.5	7.69		
C13	no proton	169.9			

Table 2 Summary of the *bs*MenD-catalyzed reductive C–N bond ligation between nitro compounds and 2-oxo-glutarate [a)]

Substrate	Product	Conversion (%) [b)]
	 **1**	>99
	 **2**	>99
	 **3**	>70
		>50
	no product	0
	no product	0
	no product	0
	no product	0
	no product	0
	no product	0
	no product	0

a) Reaction mixture contained 100–200 μM nitro compound, 2 mM 2-oxo-glutarate, ThDP 250 μM and 8.9 μM MenD in 50 mM Tris buffer (pH 7.8) supplemented with 50 mM NaCl and 10 mM $MgCl_2$ and was incubated at 25 °C for 20 h. 2-Oxo-glutarate was replenished every 2 hours; b) the conversion rate was determined by HPLC analysis as described in the Materials and methods.

(2H, t, J = 6.80 Hz), 7.45–7.66 (2H, m, aromatic), 7.24–7.27 (2H, m, aromatic).

3.3 C–N bond ligation between nitroso compounds and 2-*oxo*-glutarate

Nitrosoarenes are structurally similar to nitroarenes and may react with 2-oxo-glutarate in the presence of *bs*MenD activity like the latter. To test this possibility, two commercially available nitroarenes, nitrosobenzene and 2-nitrosotoluene, were used in the chemoenzymatic synthesis. The reactions were similarly subjected to real-time spectroscopic monitoring as well as by HPLC analysis. As shown in Figure 3(a), 2-nitrosotoluene reacted very fast with the ketoacid, as indicated by the fast disappearance of the characteristic absorbance of the nitroso compound at 280 nm. The reaction was complete within 15 min. HPLC analysis found that a product was formed and its amount was not changed for 2 h. Similarly, very fast reaction was observed for nitrosobenzene. The products of these two reactions were purified and determined to be *N*-(2-methylphenyl) succinyl hydroxamate (**4**) and *N*-phenyl succinyl hydroxamate (**5**), respectively. The isolation yield of both products was > 60%.

Characterization data for *N*-(2-methylphenyl) succinyl hydroxamate (**4**): $C_{11}H_{13}NO_4$, calcd. 223.0845, found 224.0941 ([M + H]$^+$) and 246.0745 ([M + Na]$^+$); δ_H (400 MHz, CD_3OD): 2.3 (3H, s), 3.05 (2H, t, J = 6.80 Hz), 2.72 (2H, t, J = 2.80 Hz), 7.33 (4H, m, aromatic). Characterization data for *N*-phenyl succinyl hydroxamate (**5**): $C_{10}H_{14}NO_4$, calcd. 209.0688, found 210.0708 ([M + H]$^+$) and 232.0607 ([M + Na]$^+$); δ_H (400 MHz, CD_3OD): 3.04 (2H, s, br), 2.72 (2H, t, J = 6.80 Hz), 7.50–8.50 (5H, m, aromatic).

3.4 C–N bond ligation between nitroso compounds with other α-ketoacids

Due to the high reactivity of the nitroso compounds with 2-*oxo*-glutarate, we used them to explore the substrate specificity of *bs*MenD towards α-ketoacids. The results for six α-ketoacids are shown in Table 3. The expected hydroxamate products were detected by HPLC only for 2-*oxo*-butyrate. The *N*-(2-methylphenyl) butyryl hydroxamate (**6**) product derived from 2-nitrosotoluene has a calculated molecular weight of 179.0946, consistent with the found molecular ions at m/z = 180.1023 ([M+H]$^+$) and m/z = 201.0836 ([M+Na]$^+$). The *N*-phenyl butyryl hydroxamate (**7**) product derived from nitrosobenzene has a calculated molecular weight of 165.0790, consistent with the found molecular ions at m/z = 166.0885 ([M+H]$^+$) and m/z = 188.0720 ([M+Na]$^+$). However, the conversion rate was not determined due to decomposition of the nitroso compounds in aqueous solution after prolonged incubation. Probably due to the same instability of the nitroso compounds, no product was found for other α-ketoacids that might interact with the enzyme too slowly to afford a detectable product.

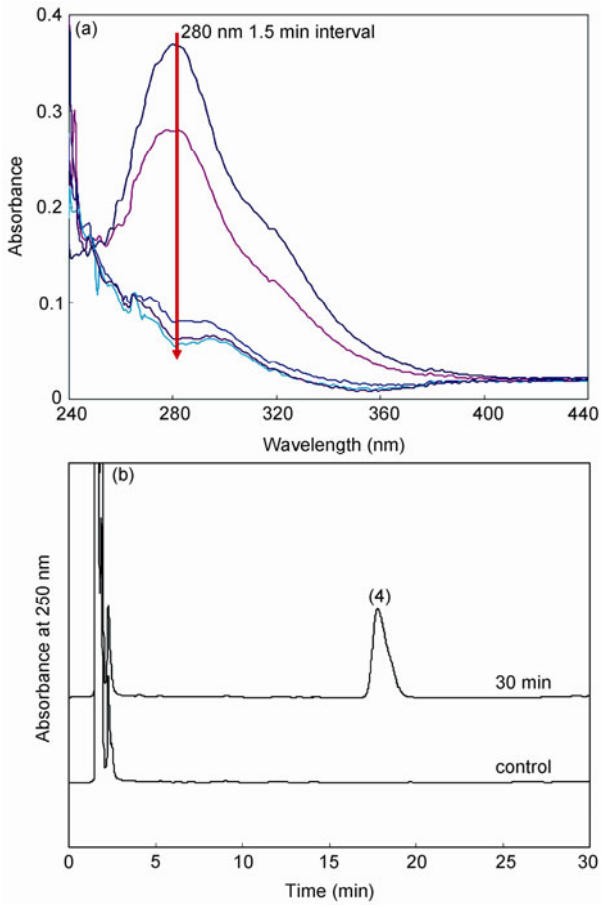
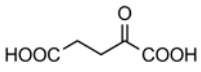
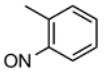
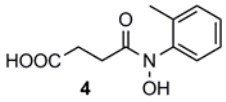
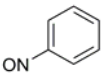
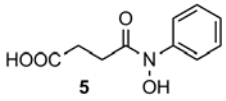
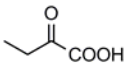
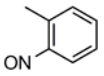
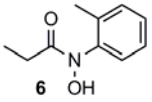
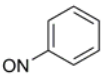
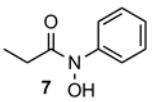
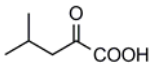
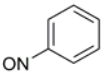
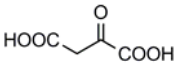
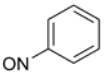
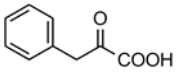
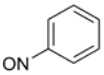
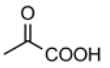
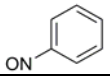


Figure 3 The *bs*MenD-catalyzed reaction of 2-nitrosotoluene and 2-oxo-glutarate. (a) Reaction of 2-nitrosotoluene and 2-ketoglutarate with UV-vis spectra taken every 1.5 min. The reaction was carried out at 25 °C in a solution containing 100 μM 2-nitrosotoluene, 3 mM 2-ketoglutarate, 100 μM ThDP and 2.68 μM *bs*MenD in 50 mM Tris buffer (pH 7.8) supplemented with 50 mM NaCl and 10 mM $MgCl_2$; (b) HPLC analysis of the reaction product at different time points. The reaction was carried out at 25 °C in a solution containing 200 μM 2-nitrosotoluene, 2 mM 2-ketoglutarate, 100 μM ThDP and 268 nM *bs*MenD in 50 mM Tris buffer (pH 7.8) supplemented with 50 mM NaCl and 10 mM $MgCl_2$. The control reaction did not contain *bs*MenD.

3.5 Discussion

The MenD orthologue from *E. coli* has been shown to possess relaxed substrate specificities in using structurally diverse α-ketoacids to generate the acyl anion intermediate for addition to carbonyl electrophiles [22]. However, the non-physiological reactions are slow, indicating that the enzyme has achieved a high level of specificity for the native substrates. This is supported by the results presented here. The observed lack of *bs*MenD activity towards cinnamic acid and its derivatives is a testament for the high

Table 3 The *bs*MenD-catalyzed C–N bond ligation between nitroso compounds and α-ketoacids [a)]

Donor	Acceptor	Product
		 **4**
		 **5**
		 **6**
		 **7**
		nd.[b)]
		nd.
		nd.
		nd.

a) Reaction mixture contained 200 μM nitrosobenzene or 2-methyl nitrosobenzene, 2 mM 2-ketoacid, 100 μM ThDP, 268 nM MenD in 50 mM Tris buffer (pH 7.8) supplemented with 50 mM NaCl and 10 mM $MgCl_2$ and was incubated at 37 °C for 6 h; b) nd = not detected.

specificity of the enzyme for isochorismate. In addition, the detected low reactivity of the non-physiological α-ketoacid substrates towards nitrosoarenes provides evidence for the stringent structural requirements on the 2-*oxo*-glutarate substrate. These experimental observations are fully consistent with the crystallographic studies in which several substrate recognition motifs have been identified in the enzyme active site for each substrate [20, 21].

It was a total surprise that *bs*MenD catalyzes formation of the *N*-alkyl hydroxamate from 4-nitrocinnamic acid and 2-*oxo*-glutarate. We propose a mechanism involving a reductive C–N bond ligation reaction as shown in Figure 4. In this mechanism, the enzyme-mediated decarboxylative addition of the 2-*oxo*-glutarate substrate with the ThDP cofactor affords the acyl anion intermediate as usual, which then reduces the oxidative nitro group in 4-nitrocinnamic through transfer of two electrons. This electron transfer oxidizes the acyl anion to acyl-thiazolium that subsequently undergoes hydrolysis to recover the enzyme cofactor, while the nitro compound is reduced to a nitroso intermediate. In the next step, the recovered ThDP cofactor converts another molecule of 2-*oxo*-glutarate to the acyl anion, which undergoes nucleophilic attack on the nitroso group of the reduction product to eventually form the *N*-alkyl hydroxamate. The proposed formation of the nitroso intermediate is strongly supported by the much faster reaction of nitrosoarenes with 2-*oxo*-glutarate (Figure 3) and 2-*oxo*-butyrate to form similar *N*-alkyl hydroxamate products.

The proposed electron transfer could occur through bond or through space. Considering that similar redox reaction also occurs to bulky electron acceptors such as 6-dichlorophenolindophenol (DCPIP) (data not shown), through-space electron transfer is more likely. However, the other possibility cannot be ruled out. Suggested by the many inactive nitro compounds listed in Table 2, this electron transfer is likely dependent on the oxidative potential of the electron acceptor, which has to be at least as high as nitrobenzene. This possibility suggests that similar electron transfer reactions should happen between the common acyl anion intermediate of all ThDP-dependent enzymes and any oxidants with an adequate oxidative potential, thereby opening up new chemoenzymatic applications for the ThDP-enzymes in redox chemistry. In addition, the observed *bs*MenD reduction of the nitro compounds also suggests that mitochondrial ThDP-dependent enzymes may also be involved in reduction of organic nitrates such as nitroglycerin to activate this class of important cardiovascular drugs in humans [28, 29].

4 Conclusions

Besides the aforementioned reduction reaction, the results presented here also demonstrate for the first time the use of nitroso compounds as electrophilic acceptor of the ThDP-

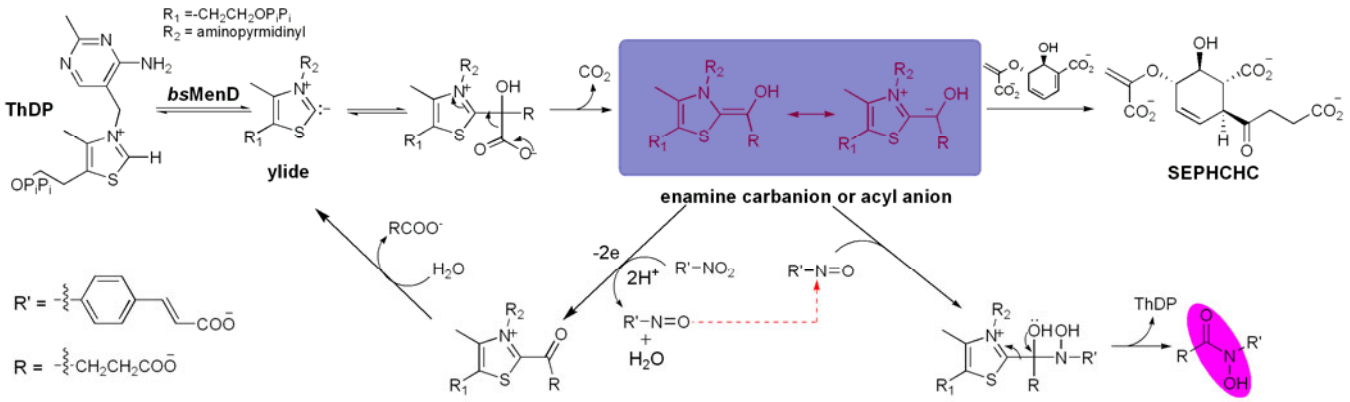


Figure 4 The proposed mechanism for the *bs*MenD-catalyzed reductive C–N bond ligation between 4-nitrocinnamic acid and 2-oxo-glutarate. SEPHCHC: (1*R*, 2*S*, 5*S*, 6*S*)-2-succinyl-5-enolpyruvyl-6-hydroxyl-3-cyclohexene-1-carboxylate; P_i: phosphate group.

derived acyl anion to form a new C–N bond. It would be interesting to see whether other ThDP-dependent enzymes could also catalyze these reactions. At present, it is not practical to use either reaction for chemoenzymatic synthesis due to the detected limitations on the substrate structure. To use these reactions, the substrate spectrum of the enzyme has to be expanded. This could be achieved by modification of the active site through site-directed mutagenesis and directed evolution, similar to the many successful examples in the literature [30–33]. Thus, in combination with modern bioengineering techniques, the new reactions found in this work may eventually lead to new chemoenzymatic applications of the ThDP-dependent enzymes.

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