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Creating Cellulose-Binding Domain Fusions of the Coenzyme A Biosynthetic Enzymes to Enable Reactor-Based Biotransformations

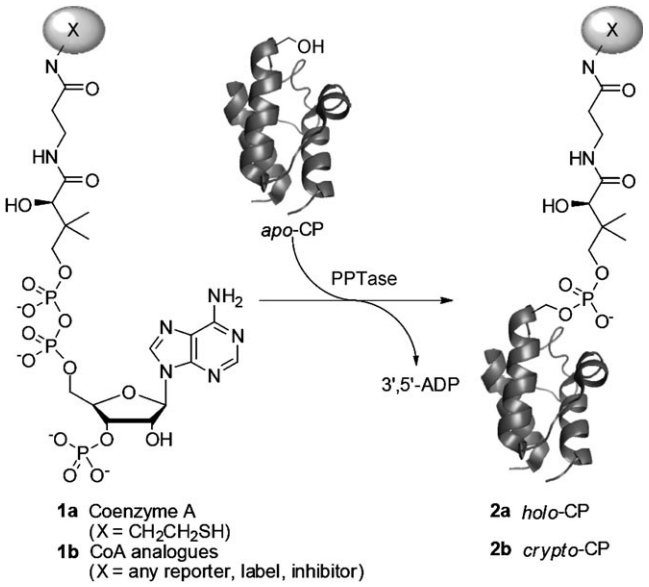
Ilse Rootman, Marianne de Villiers, Leisl A. Brand, and Erick Strauss*[a]

Coenzyme A (CoA) analogues are widely used as tools in chemical biology. Currently, most CoA analogues are prepared by biotransformation of pantothenic acid analogues using the three CoA biosynthetic enzymes (PanK, PPAT, and DPCK) that constitute the CoA salvage pathway. However, because the enzymes are usually lost or destroyed in the solution-based methods that are presently used, we created fusions of the *Escherichia coli* and *Staphylococcus aureus* PanK, PPAT, and DPCK enzymes with a cellulose-binding domain (CBD) which can be used to immobilize each of these proteins on cellulose. We show that all the CBD-fusion proteins can be expressed in soluble form, and that instead of impacting negatively on the activity of their enzyme partners, the presence of the CBD improves their kinetic profiles in some cases. We subsequently determined which combinations of the available enzymes are most effective in producing CoA and a typical CoA analogue, and used these to demonstrate that the rate of biotransformation is not severely affected even when the CBD-fusion enzymes are immobilized on cellulose. Finally, we constructed batch and column reactors from cellulose loaded with the CBD enzymes and tested these in the biocatalytic production of a fluorescent CoA analogue often used for protein labeling. Our results show that such reactors can successfully be used, and that the enzymes retain their activities upon storage in this format. This study is the first to showcase the use of a multi-enzyme reactor system based on CBD-fusion proteins in biocatalysis.

Introduction

Analogues of coenzyme A (CoA, **1a**), the universal and essential acyl carrier in biology, have found wide-spread use and application in a diverse range of biological studies.[1] For example, such analogues can be used as inhibitors of CoA-dependent enzymes or as probes of their mechanism.[2] A more recent example of its use involves the orthogonal labeling and crosslinking of carrier proteins (CPs), or more generally of any protein fused to a short carrier protein recognition sequence (Scheme 1).[3] This entails the transfer of the modified phosphopantetheine moiety of a CoA analogue that contains a fluorescent probe, affinity label, or reactive crosslinker to a conserved serine residue on the target *apo*-CP or CP sequence to form labeled *crypto*-CPs (**2b**). Such transfer reactions are usually achieved by promiscuous Sfp-type phosphopantetheinyl transferase (PPTase) enzymes. This technology, which can be applied in vitro or in vivo, has been used with great success in the investigation of CP-mediated biosynthetic pathways such as those involving fatty acid, polyketide, and non-ribosomal peptide biosynthesis.[4]

Since few CoA analogues are commercially available, their application in studies of the types listed above usually necessitates their preparation prior to use. Three general methods are currently used for this purpose.[1b] In the first method, CoA itself is derivatized, usually by reaction with a label or probe containing a thiol-reactive moiety. The second method (also referred to as the bottom-up approach) entails biotransformation of a pantothenic acid amide (a pantothenamide, **3b**) or any other pantothenic acid derivative with three of the five CoA



Scheme 1. The natural post-translational modification of *apo*-CPs by phosphopantetheinyl transferase (PPTase) enzymes to form active *holo*-CPs (**2a**) from CoA (**1a**) can also be used to form labeled *crypto*-CPs (**2b**) by transfer of the pantothenamide moiety of a CoA analogue (**1b**) containing any reporter group or affinity label.

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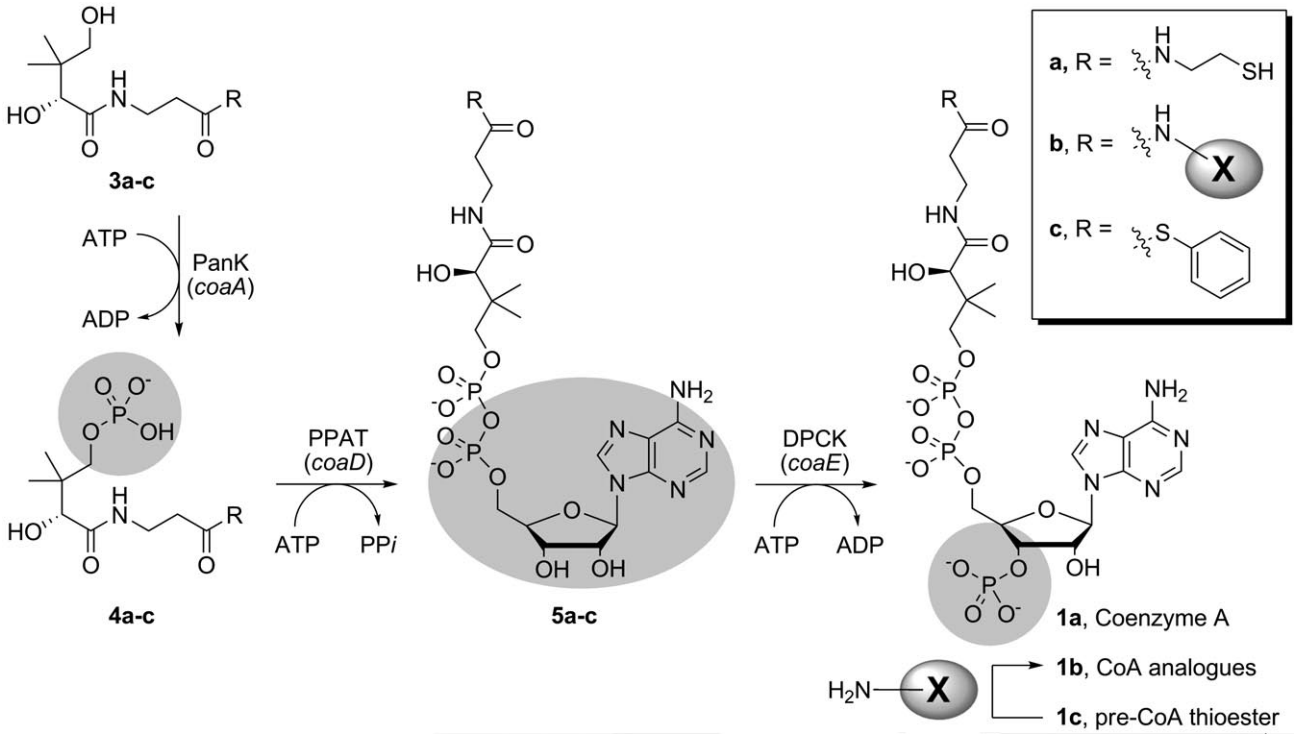
biosynthetic enzymes (i.e., pantothenate kinase, PanK), phosphopantetheine adenylyltransferase (PPAT), and dephosphocoenzyme A kinase (DPCK; Scheme 2). This biotransformation is made possible by the relaxed substrate specificity of these enzymes, which have been demonstrated to act on a very wide range of substrate analogues.[3c,5] In the last, top-down, approach a pre-CoA thioester (**1c**) is similarly prepared by biotransformation of an activated pantothenate thioester (**3c**), and subsequently treated with a primary amine containing the appropriate label or probe. The resulting aminolysis of the thioester leads to formation of the required CoA analogue.[6]

Interestingly, the biotransformation of pantothenic acid analogues by PanK, PPAT, and DPCK is rooted in a natural process, since these same three enzymes are involved in the salvage pathway of CoA biosynthesis which occurs in many organisms (Scheme 2).[1] In this pathway, the relaxed substrate specificity of PanK allows it to also phosphorylate pantetheine (**3a**), a naturally occurring pantothenamide that is formed in the degradation of CoA and *holo*-CPs. In this manner, the third intermediate in the natural biosynthetic pathway (**4a**) is formed directly without involvement of the other two remaining CoA biosynthetic enzymes, phosphopantothenoylcysteine synthetase (PPCS) and phosphopantothenoylcysteine decarboxylase (PPCDC). This salvage pathway therefore also constitutes a simple method whereby CoA may be prepared by in vitro biotransformation of pantetheine.

Currently, the *Escherichia coli* PanK (*Ec*PanK), PPAT (*Ec*PPAT), and DPCK (*Ec*DPCK) enzymes are the most popular choices for use in the biotransformation of pantothenamides or other pantothenic acid derivatives to the corresponding CoA analogues. However, the PanK of *Staphylococcus aureus* (*Sa*PanK) is also sometimes used, because unlike *Ec*PanK, it is not feedback regulated by CoA (a trait which can lead to reduced yields in one-pot biotransformations),[7] and also because its substrate selectivity has been shown to be complementary to that of *Ec*PanK.[6] The bifunctional human PPAT/DPCK enzyme has also been used successfully in some studies.[3b,8] A more in-depth discussion and analysis of these different choices is provided in a recent review of the biocatalytic production of CoA analogues.[1b]

Despite the seemingly diverse set of methods and enzymes that are available for use in the preparation of CoA analogues, all of these are still strictly employed in homogeneous solutions, which means that the CoA biosynthetic enzymes are usually destroyed upon completion of the specific biotransformation reaction in which they were used. This leads to a potentially unnecessary waste of the biocatalysts, and may also complicate the ensuing purification of the CoA product. Based on our ongoing interest in the use and application of CoA analogues, our group has therefore become interested in exploring methods whereby the PanK, PPAT, and DPCK enzymes could be immobilized on solid support. While the strategy of using immobilized CoA biosynthetic enzymes in biotransforma-



Scheme 2. The CoA salvage pathway, in which pantetheine **3a** is transformed into CoA **1a** by sequential action of the CoA biosynthetic enzymes PanK, PPAT, and DPCK (the *E. coli* gene names are given in parentheses). The groups transferred in each reaction are highlighted in grey. The same enzymes are also used to biotransform pantothenamides **3b** into CoA analogues **1b** (the bottom-up approach to CoA analogue preparation). Finally, CoA analogues can also be produced in a top-down manner by biotransformation of phenyl *S*-thiopantothenate **3c** to form the activated pre-CoA thioester **1c**, followed by its aminolysis using any suitable amine.

tions is not new (it was first applied by using partially purified PPAT and DPCK enzymes entrapped in polyacrylamide gels),[9] it has not been explored further since those first preliminary studies. Our aim in this regard was to develop an enzyme-based reactor system that could be used in either batch or column format for the production of CoA analogues from pantothenic acid derivatives. Additionally, we desired a system that would be both reusable and sufficiently stable to allow storage for several days.

Various methods of enzyme immobilization have been investigated and reported in the literature.[10] These include methods making use of covalent immobilization, ionic immobilization, hydrophobic adsorption, or encapsulation within different matrices. Although each of these methods have their own set of specific advantages and drawbacks, a disadvantage they hold in common is a requirement for the protein in question to be pure (or at least partially pure) before immobilization can be implemented. Moreover, the high cost of many of the matrices used in these techniques may also be prohibitive in regards to large scale usage. Consequently, we were keen to investigate the creation of fusion proteins between each of the CoA biosynthetic enzymes and an affinity protein or tag that would allow their purification and immobilization in a single step on an inexpensive matrix. A similar strategy, in which these enzymes were fused to the maltose binding protein (MBP), has previously been applied to facilitate their removal from one-pot biotransformation reaction mixtures that were used to modify CPs.[11] Although this demonstrated that fusion events do not impact the catalytic activity of these enzymes in solution, their ability to perform the biotransformation whilst immobilized was not investigated.

We therefore set out to examine the use of immobilized fusion proteins of the PanK, PPAT, and DPCK enzymes in the biocatalytic production of CoA analogues. We considered using the MBP-fusions employed in the study mentioned above, but decided that the cost of the amylose resin used in their affinity purification would preclude their use on a large scale. A similar, but more cost-effective alternative seemed much more attractive. Cellulose-binding domains have been used very successfully to immobilize proteins on cellulose, a low cost and abundantly available matrix that has also been approved for use in many pharmaceutical processes.[12] Immobilized CBD-fusion proteins have also successfully been used in various biocatalytic processes.[13] For example, it has been shown that the CBD fusion of *E. coli* acetohydroxyacid synthase I (AHAS I) can be used in a continuous flow reactor to produce *R*-phenylacetyl carbinol.[14] This cellulose-based reactor could be used in either batch or column mode, and was stable even in the presence of small amounts of DMSO. CBD fusions therefore seemed ideally suited for use in the construction of a multi-enzyme reactor that can similarly be used in the preparation of CoA analogues.

Herein, we describe the overexpression and catalytic characterization of fusion proteins of each of the PanK, PPAT, and DPCK enzymes from both *E. coli* and *S. aureus* with the cellulose-binding domain from the cellulosome-integrating protein CipA of *Clostridium thermocellum* (CBD_{CipA}).[15] We show that of all the possible combinations in which these fusion proteins can be used, only certain ones are productive in the biotransformation of pantothenamides. These active combinations were subsequently used to produce both natural CoA and an inhibitory CoA analogue, and a purification protocol was developed to allow the isolation and purification of the latter on multi-milligram scale. Finally, tests on the stability and reusability of the system showed that bioreactor systems containing the immobilized enzymes retained their activity for at least a week if stored at 4 °C. Taken together, this study is the first to showcase the successful use of multiple immobilized CBD-fusion enzymes for biocatalysis, and the first in which CoA analogues are produced in bioreactors.

Results and Discussion

Construction of expression vectors

Unlike the well-known maltose binding protein (MBP), CBDs are a large and diverse group of discrete protein modules (approximately 200 CBD sequences have been identified to date) that all hold a certain affinity for cellulose in common.[12a] However, they exhibit large differences in their size and sequence, as well as in the extent of their cellulose-binding affinity. Nonetheless, only a limited number of CBDs have been used in the creation of CBD fusions for affinity applications. These include the CBDs from the *Cellulomonas fimi* endoglucanase A (CBD_{cenA}) and exoglucanase/xylanase (CBD_{cex}), and the CBD from the *Clostridium cellulovorans* cellulose-binding protein A (CBD_{clos}).[13] Unfortunately these CBDs suffer from the drawback that they often lead to the formation of insoluble protein when they are heterologously expressed as fusions to a target partner. In such cases, the fusion protein must first be solubilized and then refolded in a cumbersome process before it can be used. Disappointingly, we found that both N- and C-terminal fusions of these CBDs with the *E. coli* PanK, PPAT, and DPCK proteins similarly lead to the production of insoluble proteins.

We therefore decided to prepare fusions of the CoA biosynthetic proteins with CBD_{CipA}, which has successfully been used to produce a CBD-AHAS I fusion in soluble form.[14] Towards this end, the CBD sequence from the *C. thermocellum cipA* gene, a short alternating polyproline-threonine linker-encoding sequence and a sequence encoding a tobacco etch virus (TEV) protease cleavage site were inserted between the NdeI and BamHI sites of the T7 promoter-based pET28a(+) plasmid (Novagen). A new NdeI site was also inserted upstream of the BamHI site in the process. This resulted in the formation of a new vector named pET-CBD_{CipA}, that could be used to heterologously express any target protein with an N-terminal CBD_{CipA} fusion (Figure 1). Moreover, the resulting fusion would also have a His_6-tag for purification preceding the CBD sequence, and a TEV cleavage site following it. This allows for the expressed CBD-fusion proteins to be purified by Ni^{2+}-based affinity chromatography (IMAC) if required, a potentially handy feature, because binding of CBD_{CipA} to cellulose is considered to be irreversible. At the same time, the TEV protease cleavage

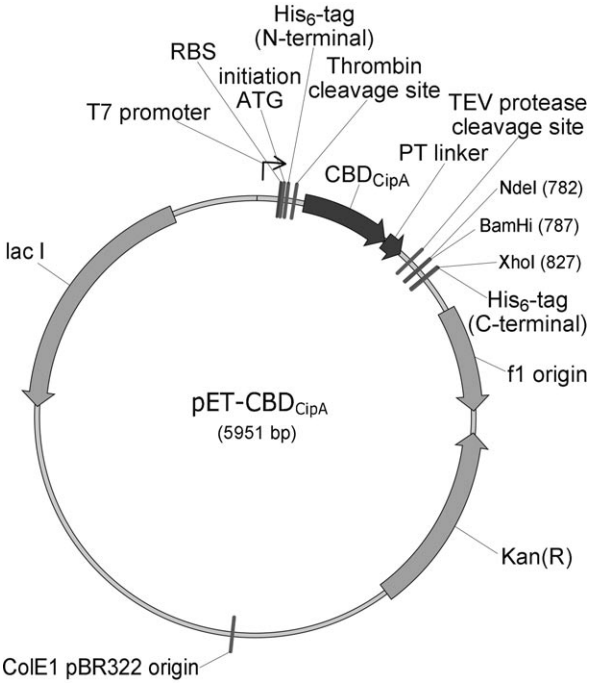


Figure 1. Plasmid diagram of the pET-CBD_{CipA} expression vector, showing its most important sequence features.

site makes it possible to conveniently and simply remove the CBD by treatment of the fusion protein with TEV protease.

Vectors that can be used for the expression of CBD_{CipA} fusions of the *E. coli* and *S. aureus* PanK, PPAT, and DPCK enzymes were subsequently successfully constructed by cloning each of the respective *E. coli* and *S. aureus coaA*, *coaD*, and *coaE* genes into the newly prepared pET-CBD_{CipA} plasmid. At the same time, the *S. aureus coaD*, and *coaE* genes were also cloned into the original pET-28a(+) to allow for expression of the His_6-tagged versions of the *Sa*PPAT and *Sa*DPCK proteins, as these enzymes were required for comparative and control purposes and their preparation has not been reported before. A His_6-tagged version of *Sa*PanK has been purified and characterized in several previous studies.[7,16]

Expression of the CBD_{CipA}-fusion proteins

With the required expression vectors in hand, we set out to conduct small-scale expression trials of the various CBD_{CipA}-fusion proteins. Various expression conditions were investigated to determine which optimally supported the formation of soluble protein. Among the conditions that were varied was the post-induction temperature (25 °C vs 37 °C), the final concentration of the inducer isopropyl β-D-1-thiogalactopyranoside (IPTG; concentrations between 50 μM and 1 mM were tested), and the type of growth medium. In regards to the latter, we specifically compared growth in Luria–Bertani (LB) medium with IPTG-based induction to growth in an auto-inducing medium (ZYM-5052).[17] This medium is known to provide high-density cultures over a wide range of conditions, and also induces expression automatically. Similar expression trials were also performed for the His_6-tagged versions of the *S. aureus* CoA biosynthetic enzymes (expression conditions for the His_6-tagged *E. coli* CoA biosynthetic enzymes have been established in previous studies).

The results of the expression trials showed that, in contrast to our previous experiences with the other CBDs, fusions of all the CoA biosynthetic enzymes with CBD_{CipA} proved to be soluble. The expression yields of the CBD fusions even surpassed that of the His_6-tagged versions in some cases. Moreover, although we found that the optimum expression conditions for each protein was slightly different, in all cases, the best yields were obtained when expressions were performed in the auto-induction medium. The optimum expression yields for all the proteins in both LB and auto-induction media, and the conditions under which they were achieved are given in Table 1.

Purification of the CBD_{CipA}-fusion proteins

To enable full characterization of the CBD_{CipA}-fusion proteins, all six fusion proteins were purified by means of Ni^{2+}-based IMAC, taking advantage of the N-terminal His_6-tag encoded by the pET-CBD_{CipA} vector. Initially, these purifications were attempted in tris(hydroxymethyl)aminomethane (Tris)-based buffers at pH 7.6. However, we found that in the presence of this buffer, the fusion proteins precipitated upon elution from the Ni-NTA affinity resin with imidazole. To overcome this problem, several alternative buffers were used in an attempt to find conditions in which the target fusion proteins remained soluble. Trial purifications were therefore conducted in Tris-HCl, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and phosphate buffers at different concentrations and pH values. Finally,

Table 1. Optimum expression conditions and expression yields for the His_6-tagged and CBD_{CipA}-fusion versions of the *E. coli* and *S. aureus* CoA biosynthetic enzymes conducted in LB and auto-induction (ZYM-5052) media.[a]

Protein	His_6-tagged proteins		CBD_{CipA}-fusion proteins	
	Yield from LB medium with IPTG induction [$mg\,L^{-1}$][a]	Yield from auto-induction medium [$mg\,L^{-1}$][b]	Yield from LB medium with IPTG induction [$mg\,L^{-1}$][[a]]	Yield from auto-induction medium [$mg\,L^{-1}$][b]
*Ec*PanK	59.4 (0.8 mM)	ND[c]	ND	45.4
*Ec*PPAT	51.8 (1.0 mM)	ND	1.8 (0.25 mM)	9.2
*Ec*DPCK	58.0 (1.0 mM)	ND	ND	28.6
*Sa*PanK	19.4 (0.5 mM)	20.4	2.4 (0.2 mM)	4.1
*Sa*PPAT	2.9 (50 μM)	12.3	ND	8.8
*Sa*DPCK	3.2 (0.1 mM)	14.8	ND	17.6

[a] For expressions conducted in LB medium and induced with IPTG, the final IPTG concentration is given in parentheses. In all cases, expression was continued for 18 h at 37 °C post-induction prior to cell harvesting, except for the His_6-tagged *E. coli* enzymes and His_6-tagged *Sa*PanK, which were harvested 3 h post-induction. [b] For expressions conducted in auto-induction medium (ZYM-5052), cultures were grown overnight. [c] ND, not determined or insoluble under these conditions.

we found that the CBD-fusion proteins could successfully be purified in soluble form by conducting the IMAC in phosphate buffer (20 mM) at pH 7.4. However, SDS-PAGE analysis of the eluted protein fraction showed that, in addition to the pure CBD-fusion protein, a smaller protein of approximately 26 kDa in size was present in these fractions (Figure 2). Interestingly, this contaminating fragment was present to some extent in all the purified CBD-fusion proteins, irrespective of the identity of the fusion partner.

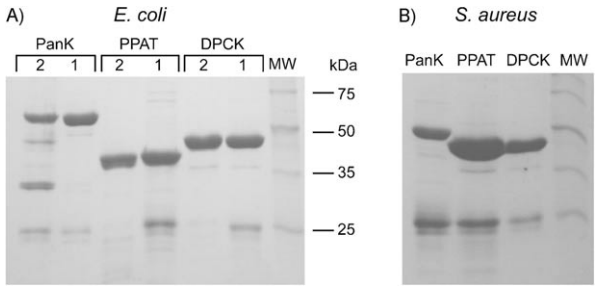


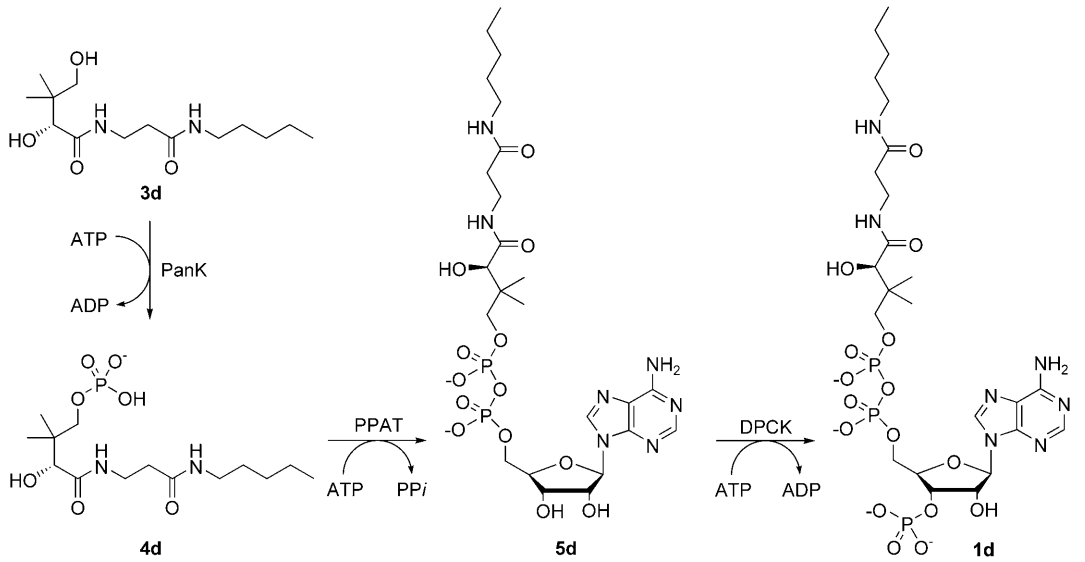
Figure 2. SDS-PAGE gel of CBD_{CipA}-fusion proteins. A) CBD_{CipA}-fusion proteins from *E. coli* after IMAC purification (step 1) and after anion exchange purification (step 2), demonstrating that the contaminating protein at about 26 kDa can be removed by anion exchange if required. B) CBD_{CipA}-fusion proteins from *S. aureus* after IMAC purification. The identity of the fusion partner proteins is indicated above each lane. MW: molecular weight markers.

Unfortunately, all attempts to remove the contaminating fragment by adjusting the imidazole gradient during protein elution failed. However, we were able to purify all the fusion proteins (with the exception of CBD-*Ec*PanK) to homogeneity by performing an additional purification step, using anion exchange chromatography on diethylaminoethyl- (DEAE-) sepharose (Figure 2A). In the case of CBD-*Ec*PanK, we found that this step leads to further degradation for reasons that are currently still unclear. However, because this particular fusion protein only contained small amounts of the contaminating fragment after IMAC purification, its determined purity of $>90\%$ was considered to be sufficient to allow confirmation of its activity.

Confirming the activity of the CBD_{CipA}-fusion proteins

With the purified protein in hand, we set out to confirm that the CBD-fusion proteins retained their native catalytic activities. This was achieved by reconstituting the CoA salvage pathway (consisting of a PanK, PPAT, and DPCK enzyme) in a step-wise fashion by using a pantothenic acid analogue, *N*-pentylpantothenamide **3d**, as primary substrate (Scheme 3). This pantothenamide was chosen because it has been shown to be accepted as an alternate substrate by the native *E. coli* CoA biosynthetic enzymes, which convert it to ethyldethia-CoA **1d**, a known anti-metabolite.[5a] Moreover, *N*-pentylpantothenamide does not have a thiol group, which eliminates the interference of possible disulfide forming side reactions. Product formation in these reactions was confirmed by both HPLC and LC–MS analyses.

We established the activity of all the newly prepared proteins by performing three different sets of reactions. In the first set, the CBD fusions of the *E. coli* PanK, PPAT, and DPCK enzymes were combined, whereas in the second, the CBD fusions of the three *S. aureus* CoA biosynthetic enzymes were used. Finally, the activity of the native (His_6-tagged) *Sa*PPAT and *Sa*DPCK enzymes, which have not previously been characterized, were confirmed by combining these enzymes with *Sa*PanK. A typical result, obtained by combining either *Sa*PanK and *Sa*PPAT, or *Sa*PanK, *Sa*PPAT, and *Sa*DPCK, is shown in Figure 3. The activity analyses of all the other combinations gave similar results. In this manner, we were able to confirm that all the newly prepared enzymes were active, and that the CBD_{CipA} module also did not inhibit the activity of the fusion



Scheme 3. Reconstituting the CoA salvage pathway with *N*-pentylpantothenamide **3d** as an alternative substrate to confirm the activity of the newly prepared enzymes.

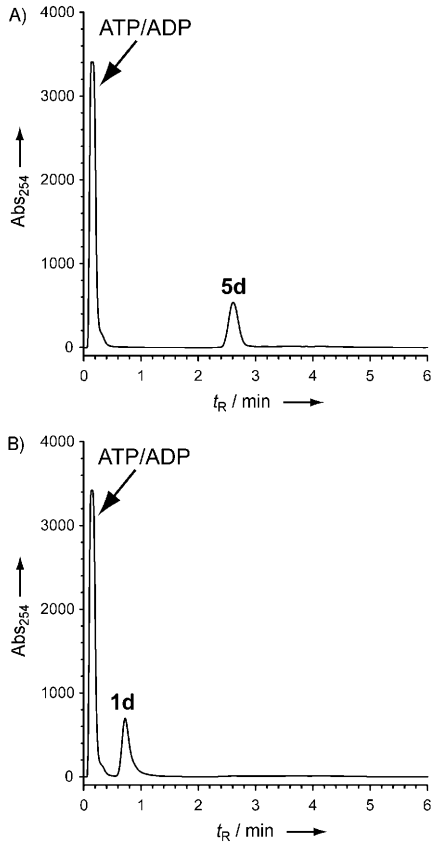


Figure 3. Confirmation of the activity of the newly prepared CoA biosynthetic enzymes by demonstrating their ability to biotransform *N*-pentylpantothenamide **3 d** (Scheme 3). A) HPLC chromatogram of a reaction mixture containing *N*-pentylpantothenamide **3 d**, ATP, *Sa*PanK, and *Sa*PPAT, showing formation of ethyldethia-dephospho-CoA **5 d**. B) HPLC chromatogram of the same reaction mixture which additionally contained *Sa*DPCK, showing formation of ethyldethia-CoA **1 d**.

partner in the CBD-fusion proteins. To establish whether the CBDs affect the activity of their fusion partners, we determined the kinetic parameters of the CBD-*Ec*PanK, CBD-*Ec*PPAT, and CBD-*Ec*DPCK enzymes acting on pantetheine **3 a**, 4′-phosphopantetheine **4 a**, and dephospho-CoA **5 a**, respectively. These data were subsequently compared to that of the corresponding native (His_6-tagged) versions. The results, shown in Table 2, indicate that in the presence of the CBD, the K_M values of the fusion proteins vary by a factor of no more than two in comparison to those of the native enzymes. More importantly, the CBD fusions all seemed to have higher activities as indicated by their increased k_{cat} values. Overall, the kinetic data clearly show that the CDB-fusion proteins either have similar or improved activity profiles compared to the His_6-tagged enzymes.

Finally, we also deemed it important to determine whether, once combined in solution, the CBD-fusion proteins showed significantly different rates of biosynthesis compared to the His_6-tagged versions. The rate of product (ethyldethia-CoA **1 d**) formation was therefore determined for a reaction catalyzed by *Ec*PanK, *Ec*PPAT, and *Ec*DPCK compared to a combination of the corresponding CBD-fusion proteins. The results show that the CBD-fusion protein combination actually forms the product slightly faster than the combination of native enzymes, although under the conditions used, both reactions were essentially complete within ten minutes (Figure 4).

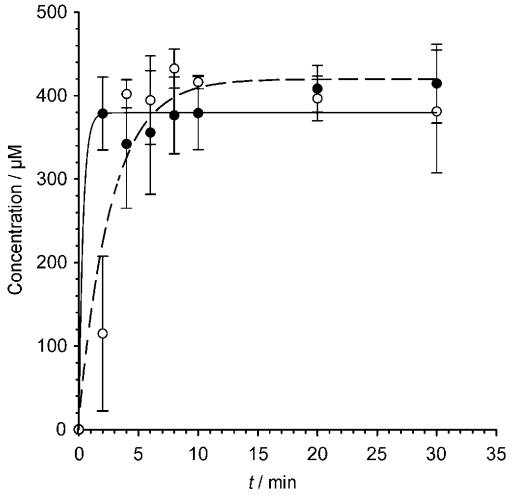


Figure 4. Comparing the rate of biosynthesis of a CoA salvage pathway reconstituted from the His_6-tagged *Ec*PanK, *Ec*PPAT, and *Ec*DPCK enzymes (open circles, dotted line) to one consisting of their CBD-fusion counterparts (filled circles, solid line). The reactions were conducted using *N*-pentylpantothenamide **3 d** (0.5 mM) as substrate. Symbols show the average amount of ethyldethia-CoA formed over time in three separate experiments, as determined by HPLC analysis. The error bars show the standard deviation. Lines were obtained by fitting the data to an equation describing an exponential rise to maximum.

Table 2. Kinetic parameters of the His_6-tagged and CBD-fusion versions of the *E. coli* CoA biosynthetic enzymes acting on the indicated substrates.

Enzyme (substrate)[a]	K_M [µM]	k_{cat} [min^{-1}]	k_{cat}/K_M [$s^{-1}mM^{-1}$]
*Ec*PanK (**3 a**)	15.5	2.27	146
CBD-*Ec*PanK (**3 a**)	38.1	5.11	134
*Ec*PPAT (**4 a**)	188	0.88	4.68
CBD-*Ec*PPAT (**4 a**)	92.2	1.42	15.43
*Ec*DPCK (**5 a**)	707	0.53	0.74
CBD-*Ec*DPCK (**5 a**)	1440	1.53	1.06

[a] Enzymes were tested against the substrates of the native CoA salvage pathway as indicated by the compound number following the name of each enzyme.

Comparison of the activity of all possible CBD_{CipA}-fusion protein combinations

After we confirmed that all the CBD-fusion proteins were active, and that the presence of the CBD did not negatively impact their activity, we wanted to determine which combination of CBD-PanK, CBD-PPAT, and CBD-DPCK enzymes gave the highest yields in a biotransformation reaction. Our aim was to identify the best combination of CoA biosynthetic enzymes for use in large-scale biotransformations.

With two proteins being available representing each enzyme activity (an *E. coli* and an *S. aureus* enzyme in each case), we were able to reconstitute the CoA salvage pathway in eight different ways. Moreover, because we were also interested in

determining the effect of using different substrates, a total of 16 different biotransformation reactions were eventually prepared by using two different substrates with each of the eight possible enzyme combinations. The two substrates were pantetheine **3a** (which should lead to the formation of CoA **1a**), and *N*-pentylpantothenamide **3d** (which should result in formation of ethyldethia-CoA **1d**). Reaction mixtures were incubated for 1 h at 37 °C, followed by determination of the amount of product formed by HPLC analysis. The results are summarized in Figure 5.

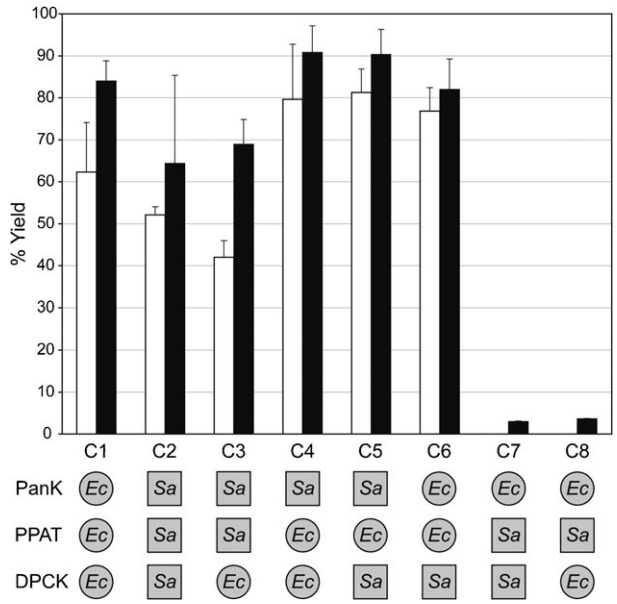


Figure 5. Comparison of the activities of the eight possible combinations (labeled C1 to C8) of the available *E. coli* and *S. aureus* CBD-fusion proteins constituting an active CoA salvage pathway using pantetheine **3a** (open bars) and *N*-pentylpantothenamide **3d** (filled bars) as substrates. The identity of each combination is figuratively represented below the graph. Each bar represents the average amount of product formed (relative to the available substrate) in three separate experiments. The error bars denote the standard deviation.

The data show that of the 16 reactions tested, 10 showed a conversion of 60% or more within the given incubation period. The highest yields were obtained with combinations that made use of *Ec*PPAT (i.e., C1, C4, C5, and C6) suggesting that *Sa*PPAT is not as active as its *E. coli* counterpart. Moreover, of the active combinations, those containing *Sa*PanK (C4 and C5) gave slightly higher yields than those making use of *Ec*PanK (C1 and C6). This may be due to the fact that only the latter enzyme is feedback regulated by CoA (and to a much lesser extent by CoA derivatives).[1b] Two combinations, C7 and C8, showed no or nearly no product formation. In both of these combinations, *Ec*PanK was combined with *Sa*PPAT, which indicates that this grouping may not result in a properly reconstituted CoA salvage pathway. Finally, all the active combinations showed greater conversion with the pantothenamide **3d** compared to the natural substrate **3a**, which is in agreement with a previous study that showed that the *E. coli* CoA biosynthetic enzymes prefer the pantothenamide-derived analogues to the natural substrates, and forms ethyldethia-CoA **1d** faster than CoA **1a**.[5a]

Since the conversion yields of combinations C1, C4, C5, and C6 with **3d** and C4, C5, and C6 with **3a** as substrate do not differ significantly, any one of these combinations seem to be excellent choices for conducting large-scale biotransformations. However, two considerations influenced our final decision as to which of these would be used in further studies. Firstly, we had to consider the potential feedback inhibition that *Ec*PanK may experience in the presence of CoA, and the effect that this may have on yield. Secondly, the different expression yields of the various CBD-fusion proteins had to be taken into account (Table 1), as large scale biotransformations would potentially also require large amounts of enzyme. In light of these factors, we therefore settled on using combination C1, which consists of all the *E. coli* enzymes, for biotransformations of any pantothenamide (**3b**, Scheme 2) as the CoA analogues (**1b**) formed in such reactions are unlikely to inhibit the activity of the CBD-*Ec*PanK enzyme.[1b] For the biocatalytic production of CoA **1a** from pantetheine **3a**, combination C6, which is made up of CBD-*Ec*PanK, CBD-*Ec*PPAT, and CBD-*Sa*DPCK, was chosen. The decision was based on the fact that CBD-*Sa*PanK (used in C4 and C5) is obtained in the lowest expression yield of all the CBD-fusion proteins. Furthermore, the differences between the conversion yields for the C4, C5, and C6 combinations acting on pantetheine **3a** were deemed to be insignificant and could not be used to justify the use of this poorly expressing enzyme even though it should be refractory to feedback inhibition.

Immobilizing the CBD_{CipA}-fusion proteins on cellulose

Although the binding of CBD_{CipA} to cellulose has been reported to be irreversible, this cannot necessarily be taken as an indication that a given amount of cellulose will bind all the CBD-fusion protein it is exposed to. We therefore determined the binding capacity of the cellulose matrix for each of the four CBD-fusion proteins that make the combinations C1 and C6. This was done by adding increasing amounts of enzyme to a constant amount of cellulose, incubating the mixture at room temperature for one hour to allow immobilization to take place and then recovering any unbound protein by centrifugation. The amount of free protein present in the supernatant was subsequently determined by using the Bradford method, and could be used to calculate the portion of fusion protein that remained bound to the cellulose. The results show that when more than 1 mg of protein was mixed with 100 mg of cellulose, the percentage of bound protein fell to approximately 80% or below for all the proteins except CBD-*Ec*PanK (Figure 6). We therefore decided to use this as the maximum ratio for immobilizing the CBD-fusion proteins on cellulose.

To determine whether immobilization had any effect on the activity of the fusion proteins, the CBD-fusion enzymes making up combinations C1 and C6 were first immobilized separately on cellulose using the ratio determined above, followed by mixing of the enzyme-bound cellulose to reconstitute the two

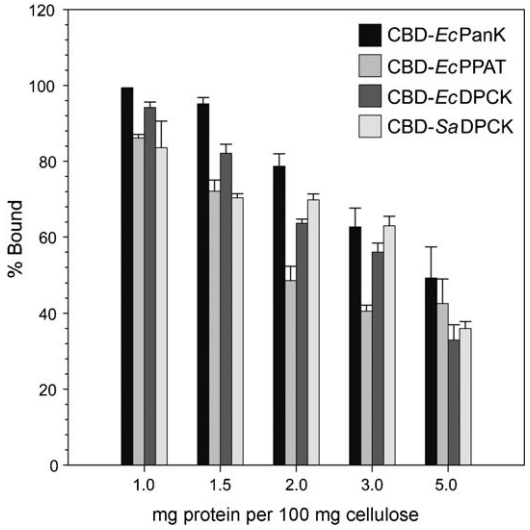


Figure 6. Binding capacity of the various CBD fusions to cellulose. The indicated amounts of each protein were separately incubated with cellulose (100 mg), followed by determination of the amount of protein that remained bound after washing (see the Experimental Section for details).

combinations. The enzymes were then used in the biotransformation of pantothenamide **3d** into **1d** and of pantetheine **3a** into CoA **1a** respectively, while the amount of product formed over time was monitored by HPLC. These data were subsequently compared to those for the same enzymes acting in solution (Figure 7).

The results show that in the case of combination C1 acting on **3d**, there is no significant difference in the rate of biotransformation whether the fusion enzymes are immobilized or not, although the final yield is slightly higher with the immobilized enzymes. This is in contrast to combination C6 acting on **3a**, which shows that immobilization severely impedes the initial rate of biotransformation. Nonetheless, even with these reduced rates about 80% conversion was reached after 80 min. Whereas this is less than the approximately quantitative conversion obtained by the same enzymes in solution, it is still considerably more than the about 20% achieved when the same combination of His_6-tagged enzymes were used. This again demonstrates to what extent the presence of the CBDs increases the productivity of the CoA biosynthetic enzymes in some cases.

Constructing a batch reactor for the biocatalytic production of CoA analogues

To demonstrate that the immobilized CBD-fusion enzymes can successfully be used in the biocatalytic production of a useful CoA analogue, we set out to construct a batch reactor specifically for this purpose. We decided to test this reactor with the fluorescent pantothenamide **3e** because the CoA analogue **1e** formed by its biotransformation has been successfully used in the labeling of various CPs (Figure 8A).[3b,c] Therefore, this is an excellent example of the kind of analogue which may be produced by a CoA biosynthetic enzyme reactor on a large scale.

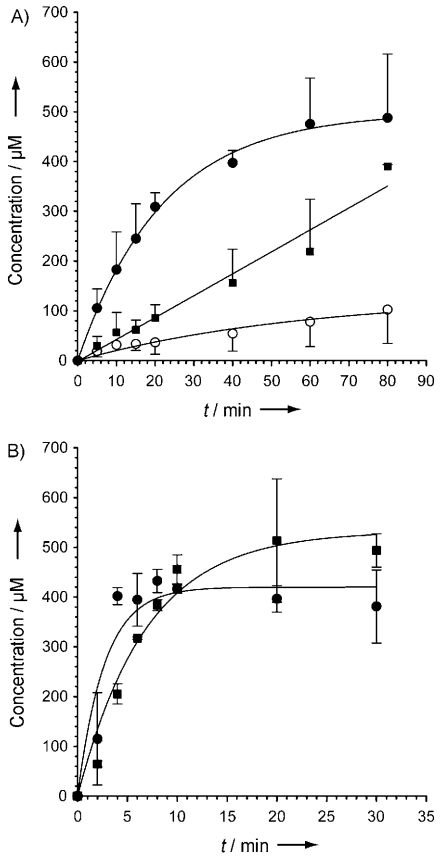


Figure 7. Comparing the rate of biosynthesis of a CBD-based CoA salvage pathway in solution and when immobilized on cellulose. A) Reactions conducted with three different versions of the *Ec*PanK, *Ec*PPAT, and *Sa*DPCK enzymes (combination C6) and pantetheine **3a** (0.5 mM) as substrate. B) Reactions conducted with two different versions of the *Ec*PanK, *Ec*PPAT, and *Ec*DPCK enzymes (combination C1) and *N*-pentylpantothenamide **3d** (0.5 mM) as substrate. The versions used were: CBD-fusion enzymes in solution (closed circles), CBD-fusion enzymes immobilized on cellulose (closed squares), and His_6-tagged enzymes in solution (open circles, only A). Symbols show the average amount of CoA or ethyldethia-CoA formed over time in three separate experiments, as determined by HPLC analysis. The error bars show the standard deviation. Lines were obtained by fitting the data to an equation describing an exponential rise to maximum, or a straight line.

Since **3e** is a pantothenamide, combination C1 was used to prepare the batch reactor, which was done by immobilizing the CBD-*Ec*PanK, CBD-*Ec*PPAT, and CBD-*Ec*DPCK enzymes directly from the crude, clarified extracts of the cells in which these proteins were overexpressed. Cellulose was added to these extracts based on the amount of each CBD-fusion protein that was estimated to be present in correspondence to the expression yields given in Table 1. The final ratio of CBD-fusion protein to cellulose was 1:100 in all cases, as this value corresponds to the optimum binding ratio determined in the previous experiment. The protein/cellulose mixture was incubated for 1 hour at room temperature, after which the immobilized protein was collected by centrifugation.

The batch reactor was constructed by addition of measured portions of each immobilized enzyme in a reaction mixture containing pantothenamide **3e** and ATP in an Eppendorf tube in a total volume of 1.5 mL. The reaction mixture was incubat-

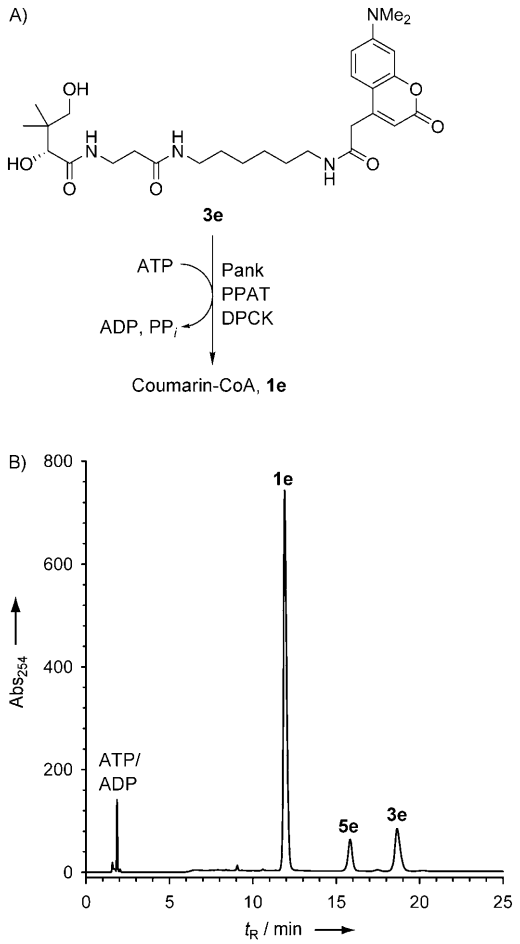


Figure 8. Production of the coumarin-CoA analogue **1e** using a batch reactor. A) Biotransformation of the coumarin-containing pantothenamide **3e** into coumarin-CoA **1e** by the CoA salvage pathway. B) HPLC analysis of the product mixture recovered from the batch reactor reaction, after purification by SPE. The peak labeled **5e** corresponds to the dephospho-CoA analogue of coumarin-CoA.

ed at 37 °C for 2 h with gentle agitation to ensure continuous contact between the substrates and the immobilized enzymes. HPLC analysis of the crude reaction mixture performed at this point showed that most of the substrate had been converted to the corresponding CoA analogue (**1e**). To further confirm its formation, the product was purified by solid phase extraction (SPE). This was achieved by removing the immobilized enzymes by conducting several centrifugation/washing cycles, followed by loading of the combined supernatants on a pre-equilibrated C_{18} SPE cartridge. The cartridge was subsequently washed with acetonitrile (3%) in NH_4OAc (10 mM) at pH 6, followed by elution of the CoA analogue by increasing the acetonitrile content to 40%. HPLC analysis of the combined product-containing fractions showed that the purified fraction mainly contained the desired analogue **1e**, although small amounts of unreacted pantothenamide **3e** and the corresponding dephospho-CoA analogue **5e** were also present. Based on this analysis and the dry weight of the purified product mixture, the yield of CoA analogue was estimated to be about 54%.

Constructing a column reactor for conducting continuous biotransformations

Encouraged by the successful preparation of a working batch reactor, we set out to construct a column reactor consisting of the same combination of CBD-fusion proteins. However, we were immediately confronted by the increased complexity of such an endeavor, which requires the consideration of additional parameters such as column dimensions, flow rate, and the manner in which the immobilized enzymes are to be packed within the column. After some initial trials, we decided on the construction of a column 10 mm × 50 mm in size. Its total volume of approximately 5 mL was made up by combining equal amounts (ca. 330 mg each) of cellulose loaded with each of the three CBD-fusion proteins making up combination C1, and mixing it with protein-free cellulose to make up the remainder of its contents. In this manner, the enzymes were equally distributed throughout the column.

To test the column reactor we decided to mimic the reaction conditions used in the batch reactor as closely as possible. A 50 mL reaction mixture containing fluorescent pantothenamide **3e** (0.5 mM) and MgATP was therefore prepared, and slowly pumped through the column to allow the biotransformation to take place. Because we expected that the amount of enzyme used in the column reactor would completely convert all substrate to product within 10 min of incubation at 37 °C, the flow rate was maintained at 0.5 mL min^{-1} to ensure a minimum residence time of 10 min in total. The column eluate was collected and analyzed by HPLC. This analysis showed complete disappearance of the starting material and formation of coumarin-CoA **1e** and its dephospho analogue **5e** in a ratio of 9:1 (Figure 9A). The eluate was subsequently purified by SPE as before, and in this manner, purified product mixture (25.6 mg) was obtained. HPLC analysis confirmed that the products **1e** and **5e** maintained a ratio of 9:1 even after purification. Taken together, the yield of CoA analogue **1e** was therefore estimated to be 84%.

To establish whether the CBD-fusion enzymes contained in the column reactor retained their activities over time, the same column reactor was stored at 4 °C and used one week later to perform exactly the same biotransformation reaction. HPLC analysis of the retrieved eluate showed that the starting material was again completely consumed, and that CoA **1e** and dephospho-CoA **5e** were formed as products. However, the ratio of these compounds decreased slightly to 83:17. Subsequent SPE purification led to the recovery of a product mixture (21 mg, 77%) similar to that obtained previously. This suggests that the PanK and PPAT enzymes remained stable over the period of storage, but that the DPCK enzyme was slowly losing activity.

These results demonstrate that the CBD fusions of the CoA biosynthetic enzymes can successfully be immobilized on cellulose and used in a batch or column reactor format to convert pantothenic acid analogues to their corresponding CoA analogues, which can subsequently be purified by SPE.

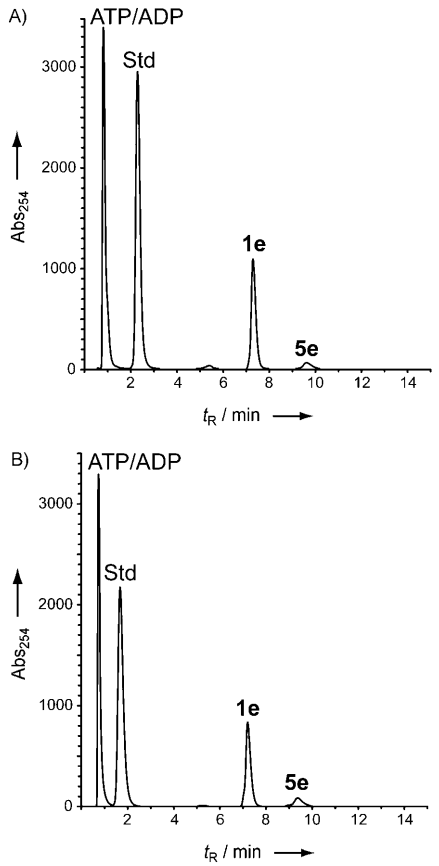


Figure 9. Production of the coumarin-CoA analogue **1e** using a column reactor. A) HPLC analysis of the product mixture recovered from a newly prepared column reactor (prior to purification). B) HPLC analysis of the product mixture recovered from the same column reactor used one week later (also prior to purification). The peak labeled **5e** corresponds to the dephospho-CoA analogue of coumarin-CoA **1e**.

Conclusions

In this study we have demonstrated that fusion of the CoA biosynthetic enzymes PanK, PPAT, and DPCK from *E. coli* and *S. aureus* to CBDs is possible, and that such fusions can be expressed in soluble form. Moreover, we have shown that the CBD module does not negatively affect the activity of these enzymes in solution or when immobilized on cellulose. In some cases, the fusion enzymes even had improved kinetic profiles compared to their His_6-tagged counterparts. Our results also indicated which of the eight possible combinations of the available enzymes give the optimum yields for the biocatalytic production of CoA and a typical CoA analogue. After having determined the binding capacity of cellulose for the fusion proteins, we were able to construct and use both batch and column reactors to successfully prepare a fluorescent CoA analogue that is commonly used in protein labeling.

Although much work can still be done to optimize the construction and use of the column reactor in regards to the flow rate, composition, ratio, and packing of the different enzymes (work that we are currently pursuing), we are confident that this study has demonstrated the practical utility and potential of these CBD-fusion proteins in the preparation of CoA analogues. Furthermore, the same technology should also be appropriate for use in other multi-enzyme biotransformations, and we hope that this study has laid a solid foundation for future work in this regard.

Experimental Section

Materials and Methods

All HPLC analyses were conducted on an Agilent 1100 series instrument using Supelcosil LC-18-T 5 μm columns (25 cm×4.6 mm or 15 cm×4.6 mm) with Supelguard LC-18-T guard columns (Supelco). Prepacked C18 STRATA-SPE cartridges (1 g and 10 g) were from Phenomenex. All protein purification procedures were conducted on an ÄKTA prime system using columns from GE Healthcare Life Science. All ESI–MS and LC–MS analyses were done at the Central Analytical Facility (CAF) at Stellenbosch University using a Waters 2690 separations module with a Waters 996 photodiode array detector for LC separations, followed by mass analysis on a Waters micromass quattro mass spectrometer. All chemicals were from Sigma or Aldrich and were of the highest available purity. The cellulose used for immobilization was Avicel PH-101 from Fluka.

Construction of the pET-CBD_{CipA} expression vector

The CBD- and following P/T-linker sequences were amplified from the pCip14 plasmid[15a,b] with the following primers (bases in capital letters introduced non-plasmid-based sequences): 5′-T ATg gca aat aca ccg gta tca ggc aat ttg aag gt-3′ (forward) and 5′-ATA CAG GTT Ttc tga cgg cgg tat tgt tg-3′ (reverse). After purification of the amplified product, a second round of amplification with the following primers introduced an AseI site at the 5′-end of the PCR product, NdeI and BamHI sites at its 3′-end, and the sequence encoding the TEV protease cleavage site (all new restriction endonuclease sites are underlined): 5′-GGG GGG *ATT AAT* gca aat aca ccg-3′ (forward) and 5′-GGG GGG *GGA TCC CAT ATG* TCC CTG AAA ATA CAG GTT Ttc-3′ (reverse). The resulting amplification product was treated with AseI and BamHI, and the digested product was ligated to pET-28a(+) (Novagen) that had previously been treated with NdeI and BamHI. This ligation was made possible because AseI and NdeI create complementary ends, which, once ligated, cannot be cleaved by either enzyme. The resulting plasmid was renamed pET-CBD_{CipA}.

Construction of CBD-fusion and His_6-tag expression vectors

pET-28a(+) expression vectors containing the *E. coli coaA*, *coaD*, and *coaE* and *S. aureus coaA* genes were available in our laboratory;[5a,16b] these genes were excised and subcloned into the pET-CBD_{CipA} vector using NdeI and XhoI to give the pET-CBD-*EccoaA*, pET-CBD-*EccoaD*, pET-CBD-*EccoaE*, and pET-CBD-*SacoaE* plasmids. The *S. aureus coaD* and *coaE* genes were PCR amplified from genomic DNA, introducing an NdeI site upstream and an XhoI site downstream of the genes. The PCR products were subsequently digested with NdeI and XhoI and ligated to a pre-cut pET-28a(+) vector to give pET28a-*SacoaD* and pET28a-*SacoaE*, and to a pre-cut pET-CBD_{CipA} vector to give pET-CBD-*SacoaD* and pET-CBD-*SacoaE* respectively.

Overexpression and purification of proteins

All the expression vectors were transformed into BL21 Star (DE3) (Invitrogen) competent cells for expression. Expressions were performed by preparing overnight starter cultures (5 mL) in LB broth supplemented with kanamycin sulphate (30 mg L^{-1}), which was used to inoculate 500 mL of complex auto-induction media (ZYM-5052)[17] supplemented with kanamycin sulphate. Growth was continued overnight at 37 °C. Cells were harvested by centrifugation at 4600 rpm for 30 min and stored at −80 °C until needed. Frozen cell pellets were resuspended in sonication buffer (20 mM potassium phosphate, 500 mM NaCl, pH 7.4) and disrupted by sonication in 6×60 second cycles. Cell debris were removed by centrifugation at 19000 rpm for 30 min. The supernatant was loaded onto a HisTrap (1 mL) column prepared according to the manufacturer's instructions. The column was washed using sonication buffer and sonication buffer containing 75 mM imidazole, successively. The protein of interest was eluted by increasing the imidazole concentration to 500 mM. Protein-containing fractions were combined and loaded onto a 5 mL HiTrap desalting column and eluted with gel filtration buffer (25 mM potassium phosphate, 5 mM $MgCl_2$, pH 7.4). Proteins were stored at −80 °C after addition of 5% glycerol.

If required, proteins were purified by anion exchange chromatography on a 16/10 DEAE FF column. The column was equilibrated with binding buffer (50 mL, 20 mM potassium phosphate, pH 7.4) before the protein was loaded. The column was washed with binding buffer (20 mL), and the protein was eluted with a linear gradient of NaCl (200 mL, 500 mM final). Protein-containing fractions were combined and loaded onto a 5 mL HiTrap desalting column and eluted with gel filtration buffer (25 mM potassium phosphate, 5 mM $MgCl_2$, pH 7.4). Proteins were stored at −80 °C after addition of 5% glycerol.

Protein purity was confirmed by 12% SDS-PAGE. The purity of CBD-*Ec*PanK determined by electronic analysis of the SDS-PAGE gel using the gel digitizing software UN-SCAN-IT gel 6.1.

Confirming the activity of the CBD-fusion proteins

Three sets of three reactions each were conducted in total to confirm the activity of each enzyme and the formation of all the reaction intermediates. The first set consisted of the *E. coli* CBD-fusion enzymes, the second of the *S. aureus* CBD-fusion enzymes and the last of the His_6-tagged CBD-fusion enzymes. In each set, the first reaction contained only PanK, the second contained PanK and PPAT, and the third contained PanK, PPAT, and DPCK. Each reaction mixture contained Tris-HCl (50 mM, pH 7.6), ATP (5 mM), $MgCl_2$ (5 mM), DTT (2 mM), and the required enzyme (15 μg) in a final reaction volume of 150 μL. The reactions were initiated by addition of *N*-pentylpantothenamide **3d**[5a] (0.5 mM) and incubated for 20 min at 37 °C. All the reactions were quenched by transferring the tubes to 95 °C for 5 min. The precipitated protein was subsequently removed by centrifugation for 5 min at 13000 rpm before the supernatant was analyzed by HPLC.

HPLC analysis was performed using acetonitrile (5%) in NH_4OAc (100 mM, pH 6.6) as eluant with a flow rate of 1 mL min^{-1}. The amount of acetonitrile was increased over time as follows: 0–1 min, isocratic at 5%; 1–3 min, gradient from 5% to 40%; 3–8 min, isocratic at 40%; 8–10 min, gradient from 40% to 80%; 10–15 min, isocratic at 80%. Elution was monitored at 254 nm. The measured retention times were: ATP, 0.1 min; 3′-dephospho-ethyldethia-CoA **5d**, 2.6 min; ethyldethia-CoA **1d**, 0.7 min.

LC–MS analysis was performed using acetonitrile (5%) in NH_4OAc (100 mM, pH 6.6) as eluant with a flow rate of 1 mL min^{-1}. The amount of acetonitrile was increased over time as follows: 0–3 min, isocratic at 5%; 3–6 min, gradient from 5% to 40%; 6–10 min, isocratic at 40%; 10–11 min, gradient from 40% to 60%; 11–15 min, isocratic at 60%; 15–16 min, gradient from 60% to 80%; 16–18 min, isocratic at 80%. Elution was monitored at 220 nm or 254 nm depending on the absorbance properties of the compounds. Peaks were assigned by analysis of the average mass spectrum (negative mode) of each peak.

Kinetic characterization of enzymes

PanK assay: Each assay contained Tris-HCl (50 mM, pH 7.6), ATP (1.5 mM), $MgCl_2$ (10 mM), KCl (20 mM), DTT (1 mM), NADH (0.3 mM), PEP (0.5 mM), pyruvate kinase (3 units), lactic dehydrogenase (3 units), the enzyme (1.5 μg), and the substrate (30 μL) in a final volume of 300 μL. The substrate concentration was varied between 0 μM and 250 μM for pantetheine **3e** and between 0 μM and 500 μM for *N*-pentylpantothenamide **3d**. Activity was monitored by the decrease in NADH concentration over 5 min. All measurements were done in triplicate.

PPAT assay: Each assay contained 60 μL pyrophosphate reagent (Sigma, P7275), Tris-HCl (50 mM, pH 7.6), ATP (1.5 mM), $MgCl_2$ (10 mM), KCl (20 mM), DTT (1.5 mM), the enzyme (1.5 μg), and substrate (15 μL) in a final volume of 150 μL. The 4′-phosphopantetheine **4a**[5a] concentration varied between 0 μM and 1 mM. Activity was monitored by the decrease in NADH concentration over 5 min. All measurements were done in triplicate.

DPCK assay: The PanK assay was used with 3′-dephospho-CoA **5a** as substrate. Its concentration was varied between 0 μM and 4 mM. All measurements were done in triplicate.

Kinetic parameters were determined by fitting the data to the Michaelis–Menten equation ($v = k_{cat}[S]/([S]+K_M)$) using nonlinear regression (Sigmaplot 9.0).

Comparing the rates of biosynthesis

Each assay contained Tris-HCl (150 mM, pH 7.6), ATP (5 mM), $MgCl_2$ (15 mM), and 25 μg each of either the His_6-tagged of CBD-fusion versions of *Ec*PanK, *Ec*PPAT, and *Ec*DPCK or *Sa*DPCK and *N*-pentylpantothenamide **3d** (0.5 mM) in a final volume of 500 μL. The mixture was incubated at 37 °C, and aliquots were taken at specific time intervals for HPLC analysis. The protein in the aliquots was precipitated by heat treatment at 95 °C for 5 min and removed by centrifugation for 5 min at 13000 rpm before the supernatant was analyzed.

For assays of the immobilized CBD-fusion proteins, each protein was first separately immobilized by adding the protein (30 μg) to cellulose (3 mg) that has previously been washed twice with potassium phosphate (20 mM), NaCl (0.5 mM), pH 7.4, followed by a 1 h incubation at room temperature. The loaded cellulose was washed again with phosphate buffer to remove unbound protein. The volume was subsequently made up to 100 μL. The 3×100 μL solutions containing the three required fusion proteins were combined, and added to the rest of the assay components (200 μL) to make up the final reaction volume of 500 μL. The reaction was subsequently treated as described above.

HPLC analysis was performed by adding an internal standard, guanosine in 0.5 M HCl (12 μL, 6 mM), to the supernatant (60 μL). After

injecting 25 μL of this solution, the column was eluted using methanol (5%) in potassium phosphate (100 mM, pH 6.5) as eluant with a flow rate of 1 mL min^{-1}. The amount of methanol was increased over time as follows: 0–3 min, isocratic at 5%; 3–10 min, gradient from 5% to 30%; 10–20 min, isocratic at 30%. Elution was monitored at 254 nm. The measured retention times were: ATP, 3.6 min; ADP, 3.8 min; AMP, 4.8 min; internal standard, 8.6 min; ethyldethia-CoA **1d**, 14.4 min.

Comparing the activity of the combinations of CBD-fusion proteins

Each assay contained Tris-HCl (50 mM, pH 7.6), ATP (5 mM), $MgCl_2$ (5 mM), DTT (2 mM), each respective CBD-fusion protein (15 μg) in a final reaction volume of 150 μL. Reactions were initiated by addition of either *N*-pentylpantothenamide **3d** (0.5 mM) or pantetheine **3e** (0.5 mM) and incubated for 60 min at 37 °C. All the reactions were quenched by transferring the tubes to 95 °C for 5 min. The precipitated protein was subsequently removed by centrifugation for 5 min at 13 000 rpm before the supernatant was analyzed by HPLC using the same procedure as above. The measured retention times were: ATP, 3.6 min; ADP, 3.8 min; AMP, 4.8 min; internal standard, 8.6 min; CoA **1a**, 9.3 min; ethyldethia-CoA **1d**, 14.4 min.

Determining the binding capacity of cellulose for the CBD-fusion proteins

Cellulose (100 mg) in 2 mL Eppendorf tubes was washed twice with potassium phosphate (20 mM, pH 7.4) containing NaCl (0.5 M). The buffer was removed, and a predetermined amount of the respective CBD-fusion proteins in the same buffer was added to the cellulose. The tubes were incubated at room temperature for 1 h, and subsequently centrifuged to collect the loaded cellulose. The concentration of the unbound protein in the supernatant was determined with a Quick Start Bradford protein assay (Bio-Rad).

Constructing the batch reactor

Expression cell pellets (500 mg) for CBD-*Ec*PanK, CBD-*Ec*PPAT, and CBD-*Ec*DPCK prepared as described before were each dissolved in potassium phosphate (10 mL of 25 mM, pH 7.4) containing EDTA (1 mM). The cells were disrupted by sonication and centrifuged at 19000 rpm for 20 min. The supernatant was added to cellulose in 2 mL Eppendorf tubes in a ratio of 10 μg of enzyme to 10 mg of cellulose, according to the average expression yields determined previously (Table 1). The tubes were incubated at room temperature for 1 h, and subsequently centrifuged at 2500 rpm for 5 min to collect the cellulose. The supernatant was removed and the loaded cellulose was washed twice with phosphate buffer. The washed cellulose was re-suspended in Tris-HCl (500 mM, pH 7.6) containing $MgCl_2$ (10 mM).

Each batch reaction mixture (1.5 mL) contained Tris-HCl (50 mM, pH 7.6), ATP (16.5 mM), $MgCl_2$ (10 mM), immobilized enzyme (250 μg), and coumarin pantothenamide **3e** (5 mM). The reactions were incubated for 120 min at 37 °C with gentle agitation, followed by centrifugation at 5000 rpm. The supernatant was collected and the cellulose was washed twice with Tris-HCl (500 μL 50 mM, pH 7.6). The supernatant and wash fractions were combined and loaded onto an activated and pre-equilibrated (3% acetonitrile in 10 mM NH_4OAc, pH 6) C18 SPE cartridge. The column was washed with acetonitrile (3%) in NH_4OAc (10 mM), pH 6, followed by elution of the product by increasing the amount of acetonitrile to 40%. The collected product was lyophilized to yield a yellow powder (2.8 mg, 54% yield). The purified product was dissolved in buffer containing 40% MeCN and analyzed by HPLC. HPLC analysis was performed using methanol (10%) in potassium phosphate (100 mM, pH 6.5) as eluant with a flow rate of 1 mL min^{-1}. The amount of methanol was increased over time as follows: 0–3 min, isocratic at 10%; 3–5 min, gradient from 10% to 35%; 5–25 min, isocratic at 35%. Elution was monitored at 254 nm. The measured retention times were: ATP/ADP, 1.9 min; coumarin-CoA **1e**, 11.9 min; dephospho-coumarin CoA, 15.4 min; coumarin pantothenamide **3e**, 18.6 min.

Constructing the column reactor

CBD-*Ec*PanK, CBD-*Ec*PPAT, and CBD-*Ec*DPCK (330 μg of each enzyme) were immobilized on cellulose (330 mg) in separate tubes using the same procedure as described for the batch reactions. The three tubes of loaded cellulose were combined and untreated cellulose was added to obtain a volume of approximately 5 mL. This mixture was suspended in Tris-HCl (50 mM, pH 7.6), $MgCl_2$ (10 mM) and loaded into a glass column. The dimensions of the final packed column were 10 mm × 50 mm. The column was connected to a pump and equilibrated with Tris-HCl (50 mL, 50 mM, pH 7.6), $MgCl_2$ (10 mM) at a flow speed of 0.5 mL min^{-1}.

A 50 mL reaction mixture containing Tris-HCl (50 mM, pH 7.6), ATP (1.65 mM), $MgCl_2$ (10 mM), and the coumarin pantothenamide **3e** (0.5 mM) was pumped through the equilibrated column at a flow speed of 0.5 mL min^{-1} while keeping the temperature constant at 37 °C. The collected product was subsequently purified by SPE as described above. After lyophilization, the product mixture was obtained as a yellow powder (25.6 mg, 93%). HPLC analysis of the product mixture was performed as above, but in the presence of an internal standard. The retention times were: ATP/ADP, 0.8 min; standard, 2.2 min; coumarin-CoA **1e**, 7.3 min; dephospho-coumarin-CoA **5e**, 9.6 min; coumarin pantothenamide **3e**, 16.8 min. Integration of the product peaks and comparison with the internal standard showed that the ratio of **1e** to **5e** was 9:1, indicating a yield of 23.0 mg (84%) for coumarin-CoA **1e**.

After completion of the reaction, the column was washed with Tris-HCl (50 mM, pH 7.6) and stored at 4 °C for 7 days. The same procedure was repeated to afford a product mixture containing **1e** and **5e** in a ratio of 83:17 (21.3 mg, 77.1%).

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