Exploiting Enzyme Promiscuity: Carboxylic Acid Reductases can Catalyze the Synthesis of Amides

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Abstract. Carboxylic acid reductases (CARs) catalyze the reduction of a broad range of carboxylic acids to aldehydes using the co-factors ATP and NADPH, and have become attractive biocatalysts for organic synthesis. Here we exploit our mechanistic understanding of CARs to expand their reaction scope, generating biocatalysts for amide bond formation from carboxylic acid and amine. After reaction engineering, CARs were found to have broad promiscuous amidation activities both for acid substrates and amines. Optimization of reaction conditions with respect to pH and temperature allowed for the synthesis of the anticonvulsant ilepcimide with up to 96% conversion. Mechanistic studies using site-directed mutagenesis suggest that amidation of the carboxylic acid proceeds *via* direct reaction of the acyl adenylate intermediate with amine nucleophiles.

Carboxylic acids are common reagents in organic synthesis, and their interconversion to other functional groups generally requires activation to reactive acyl intermediates as the key step.[1] In Nature, there are many enzymes that catalyze transformations of carboxylic acids using activated esters as intermediates.[2] A particularly interesting group are carboxylic acid reductases (CAR)[3-4] which catalyze the reduction of carboxylic acids to aldehydes via acyl adenylates and thioesters (Figure 1). Given that both activated esters are shared with other enzyme pathways, such as those of nonribosomal peptide synthetases (NRPS),[5] we were interested in exploring the promiscuity of CARs towards other nucleophiles (Figure 1). Of specific interest were amine nucleophiles that would lead to amide bond formation from acid and amine, an important challenge in organic chemistry.[6]

Our recent structural work[7] has shown that CARs are particularly good candidates for this approach: CARs are composed of three distinct protein domains: the adenylation domain, which itself is divided into a core-domain and subdomain,[7] the acyl carrier protein with the bound phosphopantetheine group and a reductase domain. The adenylation domain activates carboxylic acids by catalyzing the formation of an acyl adenylate intermediate, at the expense of ATP.[8] The activated substrate is then transferred as a thioester to the 4'-phosphopantetheine arm, bound to a carrier protein and finally to the reduction domain where NADPH is expended to reduce the substrate to the aldehyde (Figure 1).[3] CARs have a broad substrate range and a number of CARs from different organisms are now available, making them attractive for applications in biocatalysis,[9-10] such as in enzymatic cascades.[11-13]

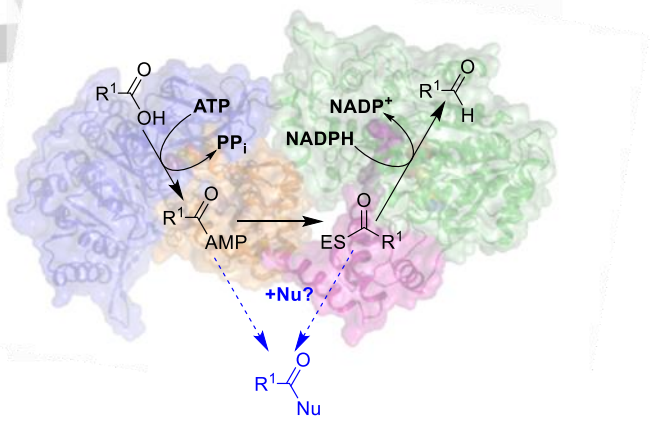

Figure 1. Carboxylic acid reductases (CAR) are composed of three domains: an adenylation domain, which is divided into a core-domain (blue) and a subdomain (orange), acyl carrier protein (pink) and a reduction domain (green). CARs catalyze reduction of the carboxylic acid at the expense of ATP and NADPH via the production of acyl adenylate and enzyme (E)-thioester intermediates. Both intermediates on the reaction pathway might potentially be intercepted by nucleophiles (Nu) such as amines (cartoon adapted from previous work[7]).

The three-domain topology of the enzyme suggests that, in the presence of ATP but the absence of NADPH, the substrate carboxylic acid might still be activated, yielding the acyl adenylate or thioester. Both intermediates could potentially react with an external nucleophile, such as an amine, to generate amides (Figure 1). Amidation would be of particular interest because many natural amide synthetases such as NRPS[14] and ATP-grasp enzymes[15-16] suffer from very narrow substrate specificity, which limits their applications in biocatalysis. Alternatively, hydrolases such as lipases[17] and proteases[18] ,

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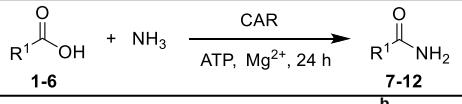
which are commonly used for amide formation[2, 19] require low water systems, such as organic solvents to drive the reaction towards amide bond formation.[20-23]

Four CAR candidates (CAR*mm* from *Mycobacterium marinum*,[3] CAR*ni* from *Nocardia iowensis*,[4] CAR*tp* from *Tsukamurella paurometabola*,[10] and CAR*se* from *Segniliparus rotundus*[24]) were produced recombinantly in *E. coli*, with the co-expressed gene for the *B. subtilis* phosphopantetheinyl transferase (PPTase) (Sfp)[25], which is required for post-translational addition of the 4'-phosphopantetheine prosthetic group.[4] The promiscuity of these purified CARs (SI) for amidation was first tested using a range of carboxylic acids **1**-**6**. Instead of NADPH, ammonia was added to the biotransformations in excess. However, initial attempts with similar reaction conditions used for CAR-mediated reductions only produced trace amounts of amide (SI).

Table 1. CARs can generate a range of primary amides **7**-**12** from acids **1**-**6** by reaction with ATP and ammonia.[a]



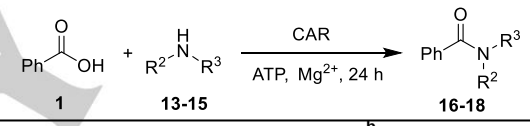
Acid	Amide	TTN[b] (conversion) CAR*mm*	CAR*ni*	CAR*tp*	CAR*se*
1	**7**	146 (11%)	36 (3%)	9 (1%)	137 (11%)
2	**8**	283 (22%)	154 (12%)	53 (4%)	99 (8%)
3	**9**	320 (25%)	167 (13%)	75 (6%)	189 (15%)
4	**10**	104 (8%)	23 (2%)	6 (1%)	5 (<1%)
5	**11**	124 (10%)	38 (3%)	19 (2%)	13 (1%)
6	**12**	174 (13%)	36 (3%)	13 (1%)	99 (8%)

a) 1 mM **1-6**, 100 mM ammonia, 100 µg mL^{-1} purified CAR, 5 mM ATP, 10 mM $MgCl_2$, 100 mM sodium carbonate buffer, pH 9.0, 37°C (30°C for CAR*se*), 24 h. b) TTN (total turnover number), conversion determined by HPLC on a non-chiral phase at 24 h.

Given that amidation reactions might have very different reaction requirements to reduction, a wider range of reaction conditions was investigated. This reaction engineering approach proved to be very successful and, in particular, increase of pH greatly increased conversion (SI) with the maximum observed at pH 9 for CAR*mm*. Formation of primary amides **7**-**12** was clearly detected by HPLC and products were identified by comparison with authentic synthetic standards. Conversions were initially recorded as total turnover numbers (TTN, moles of product formed per mole of enzyme) (Table 1) to establish catalytic activity as opposed to single turnover activation. Table 1 shows that TTN values of up to 320 were observed, demonstrating clear promiscuous catalytic activity of CARs for amidation. Higher TTNs were observed for the cinnamic acid derivatives (**2**-**3**) compared to benzoic acid derivatives (**1**,**4**-**6**). Investigations of the optimal temperature of the reactions showed different profiles for the four CARs (Table 1) (SI).

Next, amines other than ammonia were investigated for amidation of benzoic acid **1**, exploring both primary and secondary amines **13**-**15** (Table 2). Comparison of TTN values for all enzymes showed that amines **13** and **14** were better substrates than ammonia, but surprisingly propargylamine **15** only afforded amide **17** with a very modest TTN of 52 by CAR*mm*. It is interesting to note that optimal reaction temperatures varied from previous experiments with ammonia (Table 2) (SI).

Table 2. CARs can generate secondary and tertiary amides **16**-**18** by reaction of **1** with ATP and amines **13**-**15.**[a]



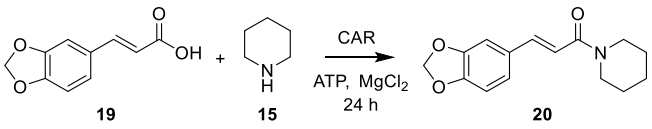
Amine	Amide	TTN[b] (conversion) CAR*mm*	CAR*ni*	CAR*tp*	CAR*se*
13	**16**	327 (25%)	154 (12%)	38 (3%)	187 (14%)
14	**17**	52 (4%)	–[c]	–[c]	–[c]
15	**18**	99 (8%)	85 (7%)	25 (2%)	102 (8%)

a) 1 mM **1**, 100 mM **16-18**, 100 µg mL^{-1} purified CAR, 5 mM ATP, 10 mM $MgCl_2$, 100 mM sodium carbonate buffer, pH 9.0, 30°C (37°C for CAR*mm*, 22°C for CAR*ni*), 24 h. b) TTN (total turnover number), conversion determined by HPLC on a non-chiral phase at 24 h. c) No activity detected.

After establishing that various carboxylic acids and amines could be used for amide production with this method, the CARs were tested as biocatalysts for the formation of a commercially-relevant target amide, ilepcimide **20**, shown to possess anti-convulsant properties.[26] Scale-up of the CAR-mediated production of ilepcimide **20** was carried out at 37°C using cell lysate containing CAR*mm*, affording 25 mg of **20** (19% yield). Further optimizations at pH 9.0 at 22°C, 30°C, and 37°C resulted

in conversions of 71% by CAR*mm* at 30°C (TTN of 923), 68% by CAR*ni* at 22°C (TTN of 883), 31% by CAR*tp* (TTN of 384) and 7% by CAR*se* (TTN of 86) both at 37°C (Table 3). Lastly, conversion of 96% to ilepcimide **20** by CAR*mm* was achieved at 30°C, pH 9.0 when the reaction was left for up to 72 h (Table 3). A pH profile was also conducted using *CARmm* for the production of **20** which confirmed that pH 9.0 was optimal for this reaction using CAR*mm*. The specific activity of CAR*mm* for the production of **20** was shown to be 12.22 ±0.15 mU mg^{-1} (SI).

Table 3. Synthesis of ilepcimide **20** by CAR amidation.[a]



	Conversion[b]			
Temperature	**CAR*mm***	**CAR*ni***	**CAR*tp***	**CAR*se***
22°C	52%	68%	5%	1%
30°C	71% (96%)[c]	50%	27%	4%
37°C	17%	48%	32%	7%

a) 1 mM **19**, 100 mM **15**, 100 µg mL^{-1} CAR, 5 mM ATP, 10 mM $MgCl_2$, 100 mM sodium carbonate buffer, pH 9.0, 22-37°C. b) Conversion determined by HPLC at 24 h. c) Value determined at 72 h.

Finally, the mechanism of amide bond formation was explored, in particular addressing the important question whether the acyl phosphate or the thioester was intercepted by the amine (Figure 1). To probe this question, the Ser689Ala variant of CAR*ni*[4] (Figure 3A) was generated. This variant lacks the Ser689 attachment site to the phosphopantetheine group that is involved in thioester formation and therefore would only allow the formation of the acyl adenylate but not the thioester. Conversion to ilepcimide **20** (79%) was still observed with this variant, suggesting that adenylation activity alone is sufficient for amidation, presumably *via* nucleophilic attack onto the acyl adenylate intermediate (Figure 3B), a reaction that is biosynthetically plausible.[27-32]

Further work was conducted using a truncated form of *CARmm,* lacking the terminal reduction domain but retaining the adenylation domain and carrier protein (CAR*mm*Δ729-1175) (Figure 3 A). Again, this variant gave a good conversion of 69% to **20**.

It should be noted that both of these CAR variants still retain amido synthetase activity, but have lost reductase activity (even in the presence of NADPH, SI) and therefore present a new enzyme family of amido synthetases with no residual CAR activity, i.e. a full switch of activity. These variants might have application as amidation catalysts in more complex enzyme cascades, both in cell-free and whole-cell systems, where the presence of NADPH would normally suppress CAR-mediated amidation in favor of reduction.[12]

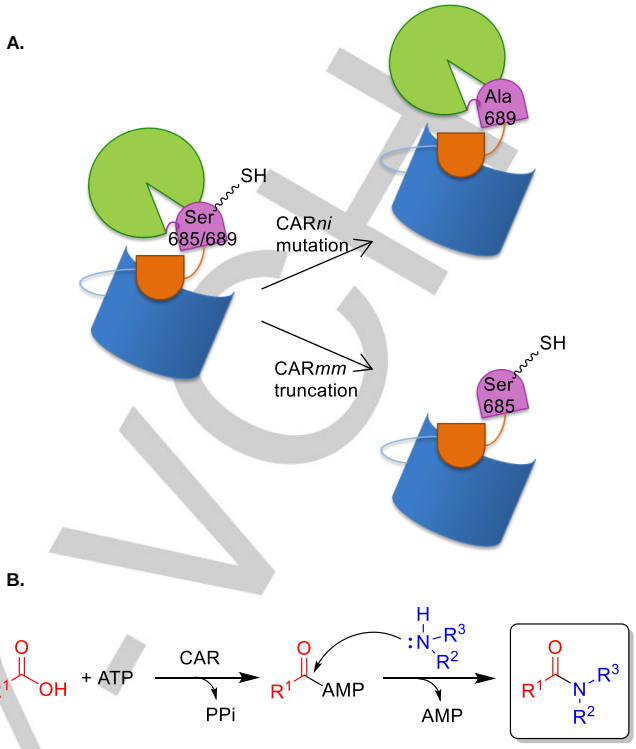


Figure 3. A) CARs are bound to a 4'-phosphopantetheine prosthetic group at serine (Ser689 on CAR*ni* or Ser685 on CAR*mm*) which is replaced with an alanine in mutant CAR*ni* Ser689Ala. A truncated version CAR*mm*Δ729-1175 lacks the reduction domain. Both variants were found to retain activity (images adapted from previous work[7]). B) Mechanism proposed for the amidation reaction.

In conclusion, we have shown that in the absence of NADPH, CARs have significant promiscuous catalytic activity for amide bond formation. The reaction was tolerant to a range of carboxylic acids and amines and could be performed in an aqueous medium using ATP as the driving force. The method was applied to a target drug molecule, ilepcimide **20** with up to 96% conversion and preparative scale-up. While our method is limited by the use of the expensive co-factor ATP, this may be overcome in future by established ATP regenerating systems.[33] Given that all four CARs tested exhibited this promiscuous activity, the range of enzymes which catalyze this amidation reaction might be rapidly expanded by tapping into the range of known CAR enzymes with differing and broad substrate specificities.[9-10] Two variants were generated by rational re-design of the CARs, which possess no reduction activity, but retain amido synthetase activity. The success with these variants suggests that the adenylation activity alone is sufficient for activation of carboxylic acids for subsequent amidation, and also lays the foundations for future work using this method in more complex enzyme systems such as in enzyme cascades or whole-cell biotransformations.

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Keywords: • amides • amidation • carboxylic acid reductase • biocatalysis • amido synthetase

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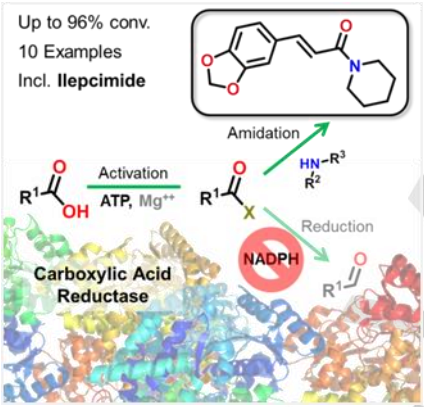
Entry for the Table of Contents

Layout 1:

COMMUNICATION

Carboxylic acid reductases can be used as biocatalysts for the activation and subsequent amidation of a range of carboxylic acids with ammonia, primary and secondary amines. The enzymes were used for the synthesis of ilepcimide.



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