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Enzymatic Activation of Lysine 2,3-Aminomutase from *Porphyromonas gingivalis*

Brian J. Brazeau,[1]* Steven J. Gort,[2] Holly J. Jessen,[2] Amy J. Andrew,[3] and Hans H. Liao[2]

Biotechnology Development Center—Eddyville, Cargill Incorporated, 1 Cargill Drive, Eddyville, Iowa[1]; Biotechnology Development Center, Cargill Incorporated, 2500 Shadywood Road, Navarre, Minnesota[2]; and Department of Biochemistry, University of Wisconsin—Madison, Madison, Wisconsin[3]

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The development of lysine 2,3-aminomutase as a robust biocatalyst hinges on the development of an in vivo activation system to trigger catalysis. This is the first report to show that, in the absence of chemical reductants, lysine 2,3-aminomutase activity is dependent upon the presence of flavodoxin, ferredoxin, or flavodoxin-NADP$^+$ reductase.

Biocatalysis has become a viable technology for the production of fine chemicals and intermediates such as β-amino acids (10, 13–15). One biocatalytic method to produce β-amino acids is via the migration of the amino group from the α-carbon to the β-carbon, catalyzed by aminomutases. The best-characterized aminomutase, lysine 2,3-aminomutase from *Clostridium subterminale* (KAM_{Cs}), is a member of the radical *S*-adenosylmethionine (SAM) superfamily and interconverts L-lysine and β-lysine (5). Variants of aminomutases engineered to have activity on other L-amino acids would be valuable biocatalysts (8). For example, a biosynthetic pathway for the production of 3-hydroxypropionic acid that includes a step in which L-alanine is converted to β-alanine is being developed using an engineered lysine 2,3-aminomutase from *Porphyromonas gingivalis* W83 (9).

A common feature of radical SAM enzymes is that a 4Fe-4S cluster in the +1 state provides reducing equivalents to cleave SAM to yield a catalytic 5′-deoxyadenosyl radical (7). The assembly of the 4Fe-4S cluster and its reduction to the +1 state are limiting factors in a biocatalytic process utilizing a radical SAM-type aminomutase. In this work we show that a reducing protein such as flavodoxin NADP$^+$ reductase (FPR), flavodoxin (FLD), or ferredoxin (FD) can activate lysine 2,3-aminomutase.

A homolog from *Porphyromonas gingivalis* W83 (KAM_{Pg}) that was 73% homologous to KAM_{Cs} was discovered by performing a BLAST search of the ERGO database (Integrated Genomics, Chicago, IL) with the KAM_{Cs} amino acid sequence (protein accession number AAD43134). The KAM_{Pg} gene was amplified from genomic DNA (ATCC BAA-308D) and cloned into pASK-IBA3 (IBA, Göttingen, Germany) using standard molecular biology techniques. The KAM_{Pg} gene was produced in *Escherichia coli* BL21(DE3) as described by the manufacturer (IBA) and was affinity purified in a Coy anaerobic chamber using the Strep-tag system. The specific activity of purified KAM_{Pg} was 30 ± 4 U/mg (mean ± standard deviation) at 37°C when assayed using the chemical reduction system described for KAM_{Cs} (1, 5, 6).

The chemical reduction method, in which the enzyme is activated via reducing equivalents provided by dithionite, has been invaluable in elegant in vitro studies of KAM_{Cs}. However, dithionite is clearly not a biological reductant. Since an industrial-scale biocatalytic process would likely include a microbial fermentation, the goal of this work was to understand the activation mechanism in vivo. Thus, it was critical to identify the proteins that may be the biologically relevant activating enzymes.

Previous studies of other members of the radical SAM superfamily have demonstrated that FLD and FPR from *E. coli* are able to activate those enzymes (2–4, 18). Since lysine 2,3-aminomutase is also a member of that superfamily, it was reasonable to expect that those proteins could activate KAM_{Pg} as well. In general, the role of FLDs is that of an electron shuttle between redox proteins. FD has been found to have a similar function; therefore, its ability to activate KAM_{Pg} was also examined.

The flavodoxin (GenBank accession number M59426) and flavodoxin NADP$^+$ reductase (accession number NC_000913) genes were amplified from *E. coli* ATCC 11303 and cloned into pASK-IBA3 using standard molecular biology techniques. The ferredoxin gene (GenBank accession number D90883) was synthesized from oligonucleotides, assembled (17), and cloned into pASK-IBA3. The genes were expressed as described by the manufacturer, and the proteins were affinity purified using the Strep-tag system. The electron transfer activity of these proteins was confirmed by assaying the diaphorase activity with NADPH as the electron donor and cytochrome *c* as the electron acceptor (16).

To activate KAM_{Pg} enzymatically, either FPR, FD, or FLD was added to as-isolated KAM_{Pg} in 50 mM HEPPS [4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; pH 8] at 37°C. The assay also contained 5 μM KAM_{Pg}, 200 μM *S*-adenosylmethionine, 50 mM L-lysine, 100 μM pyridoxal-5-phosphate, 1 mM NADPH, and 1 mM dithiothreitol. A total of 13.5 U of glucose dehydrogenase and 10 mM glucose were added as an NADPH regeneration system. The reactions were quenched with formic acid at specific time points and analyzed

* Corresponding author. Mailing address: Biotechnology Development Center—Eddyville, Cargill Incorporated, 1 Cargill Drive, Eddyville, IA 52553. Phone: (641) 969-7045. Fax: (641) 969-3850. E-mail: brian_brazeau@cargill.com.

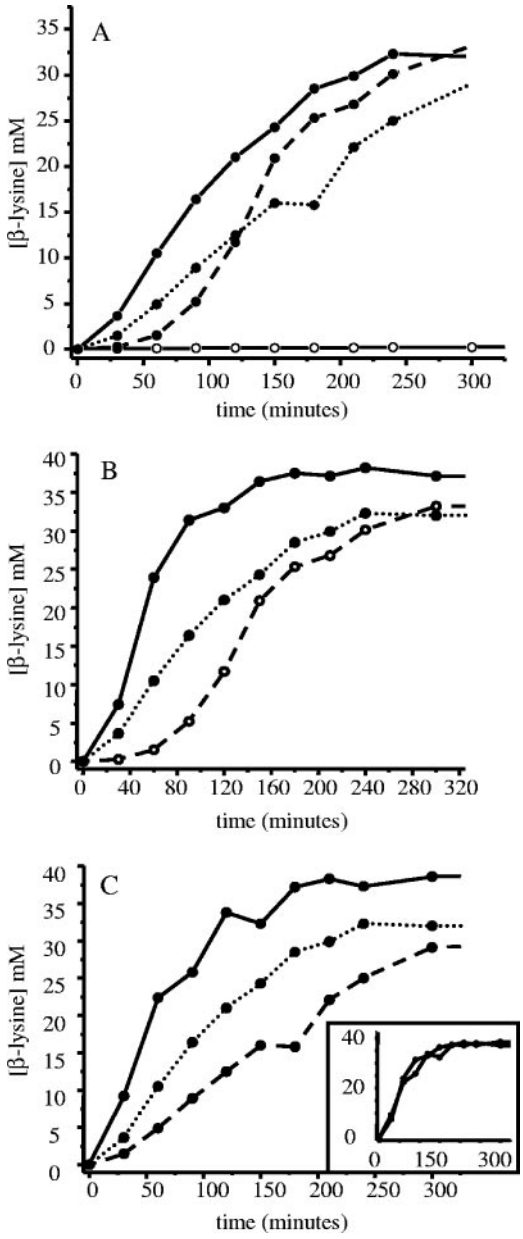

FIG. 1. Effect of electron transfer proteins on activity of KAM_{Pg}. (A) A 100 μM concentration of FPR (solid line, solid circles), FLD (dashed line, solid circles), or FD (dotted line, solid circles) was added individually to as-isolated KAM_{Pg}. The data for KAM_{Pg} with no electron transfer protein added is also shown (solid line, open circles). (B) A 100 μM concentration of FLD (dashed line, open circles), FPR (dotted line, solid circles), or 100 μM FLD plus 100 μM FPR (solid line, solid circles) was added to as-isolated KAM_{Pg}. (C) A 100 μM concentration of FD (dashed line, solid circles), FPR (dotted line, solid circles), or 100 μM FD plus 100 μM FPR (solid line, solid circles) was added to as-isolated KAM_{Pg}. The inset shows an overlay of 100 μM FLD plus 100 μM FPR and 100 μM FD plus 100 μM FPR. Each assay was performed as described in the text and was repeated at least three times. The lines through the data points do not represent the fit of a kinetic model; they are included only for clarity.

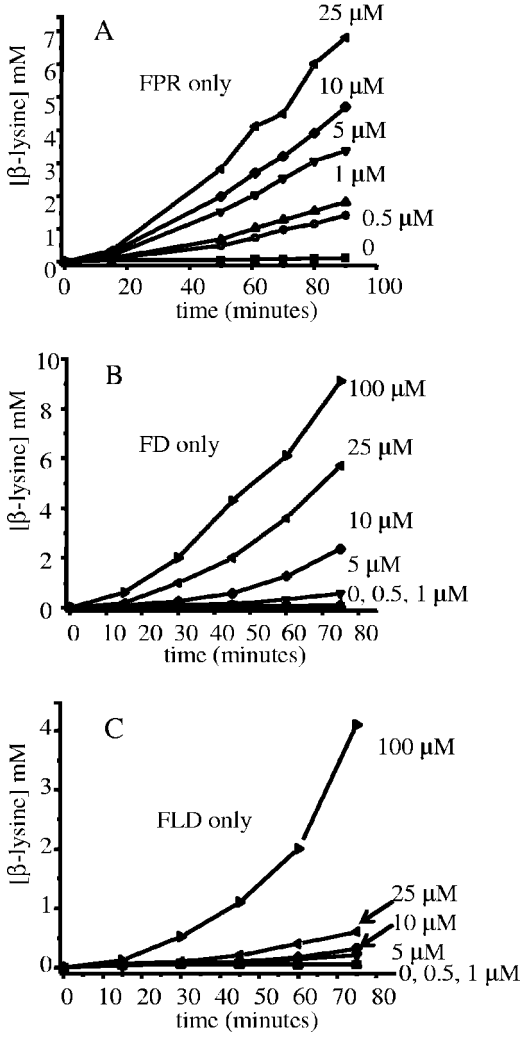

FIG. 2. The activity of KAM_{Pg} is dependent upon the concentration of each electron transfer protein. Various concentrations of FPR (A), FD (B), and FLD (C) were used. The concentration of KAM_{Pg} was 5 μM. All assays were performed as described in the text and were repeated at least three times. The lines through the data points do not represent the fit of a kinetic model; they are included only for clarity.

for β-lysine by high-pressure liquid chromatography using a cation-exchange column followed by postcolumn derivatization with *o*-phthaldehyde (Chromtech, Apple Valley, MN). The error in the assays was 10%, which was not surprising due to variations in iron and sulfide content from different enzyme purifications.

As shown in Fig. 1A, either FPR, FLD, or FD was absolutely required for the production of β-lysine by KAM_{Pg} when no chemical reductants were used. The initial rate of β-lysine production by KAM_{Pg} was much higher when FPR was used than when FD or FLD was used. This may reflect the need to initially reduce FD or FLD with NADPH before these proteins can in turn reduce the 4Fe-4S clusters of KAM_{Pg}. Combining either FLD or FD with FPR dramatically increased the rate of β-lysine production compared to when each of the electron transfer proteins was used alone, as shown in Fig. 1B and C, respectively. These results are consistent with the hypothesis that activation of KAM_{Pg} requires reduced forms of FLD or FD.

Figure 2 demonstrates that the rate of production of β-lysine by KAM_{Pg} is dependent upon the concentration of the electron

transfer proteins. These results are similar to those that have been previously reported for biotin synthase (12). However, in the case of biotin synthase, FPR was absolutely required and neither FD nor FLD alone was able to support biotin production.

A specific activity of 2.7 ± 0.4 U/mg for β-lysine production when FLD and FD were used with FPR was obtained by fitting the linear portion of the time-course graph in Fig. 1B and C. This represents a 10-fold-lower specific activity of KAM_{Pg} than that from the chemical reduction method and is likely due to the use of the KAM_{Pg} as isolated, without reconstitution with iron and sulfur prior to the assay. Previous studies with KAM_{Cs} have shown that the specific activity increases following iron reconstitution (6, 11). Because care was taken to avoid damage to the KAM_{Pg} protein by purification under anaerobic conditions, our hypothesis is that this preparation reflects the iron and sulfide content of the enzyme in vivo and is a more accurate reflection of its activity in the cell.

To the best of our knowledge, this is the first report to demonstrate that lysine 2,3-aminomutase can be enzymatically activated. The identification of these electron transfer proteins as activators of lysine 2,3-aminomutase advances our understanding of the in vivo catalytic activity of this important enzyme.

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