Biocatalytic Potential of alpha-Ketoglutarate Dependent Dioxygenases

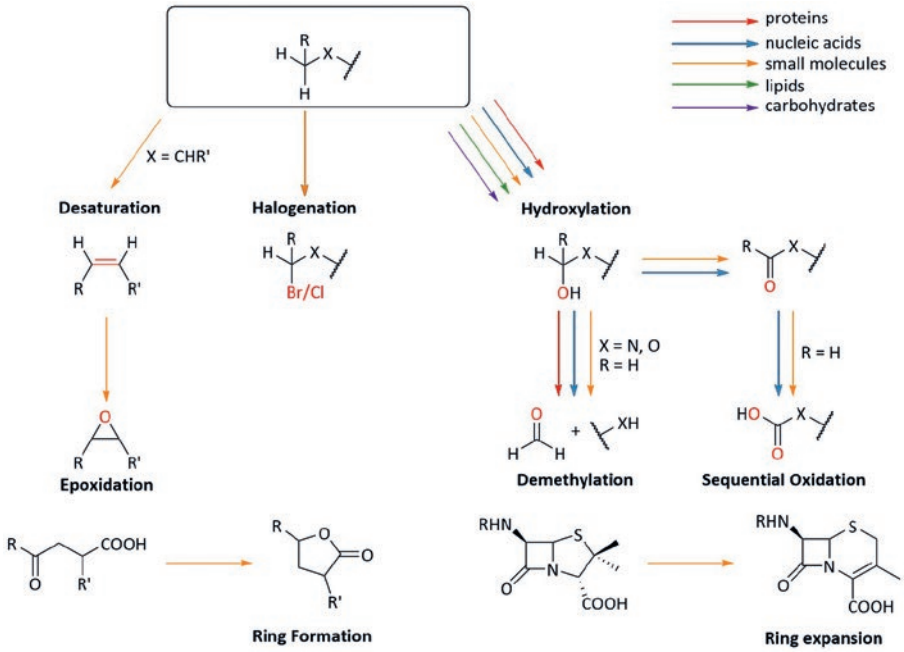
Jan Taubitz started his career with an apprenticeship as a chemical laboratory technician with integrated baccalaureate at the Novartis Pharma AG in Basel. After completion of his apprenticeship, he commenced a bachelor's degree in Chemistry at the Institute for Chemistry and Biotechnology of the Zurich University of Applied Science. Finishing his bachelor's degree with a thesis at the Competence Centre of Biocatalysis (CCBIO) of Prof. Dr. Rebecca Buller, he decided to continue his studies with a Master of Science in Chemistry. Under the supervision of Prof. Dr. Rebecca Buller and Dr. Fabian Meyer, Jan Taubitz completed his degree with a thesis about the biocatalytic potential of α-ketoglutarate dependent dioxygenases (αKGDs). Currently Jan Taubitz carries out an internship in biocatalysis at Givaudan SA in Kemptthal.

Author: Jan Taubitz

The enzyme family of α-ketoglutarate dependent dioxygenases (αKGDs) was first described in the late 1960s. Today, over 80 human αKGDs have been described and even more exist in plants and animals. αKGDs constitute the largest en-

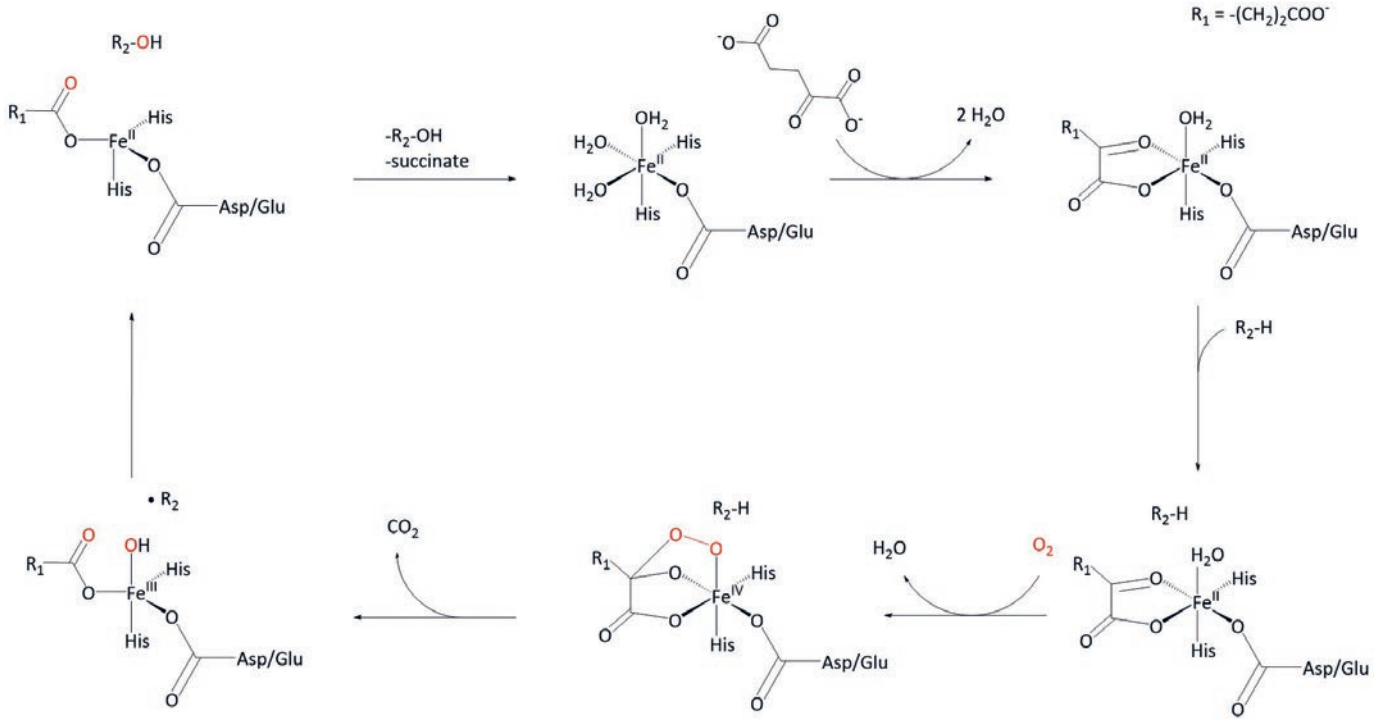
zyme family known to catalyse oxidation reactions without containing a Haem-Group [1, 2]. Notably, the reaction scope of αKGDs is versatile and includes reactions such as hydroxylations, epoxidations, halogenations and many more [3].

As the stereo- and regioselective functionalisation of unactivated C-H bonds by conventional chemical reactions remains challenging, enzymatic approaches have attracted increasing interest. The diverse reaction- and substrate scope of αKGDs makes them ideally suited candidates as biocatalysts for modifications of sp3 C-atoms in molecules. Currently, biocatalytic reactions with αKGDs catalysts are usually monitored by analysis of the substrates and products, for example by LC- or GC-MS. The diverse substrate- and reaction scope requires that the analysis method must be tailored to each enzyme specifically, a process which is time-and resource consuming. However, despite the broad substrate scope and the plethora of reactions catalysed by αKGDs, the general reaction mechanism in αKGDs is conserved and is coupled to the oxidative decarboxylation of the co-substrate α-ketoglutarate to form succinic acid in all cases [3].

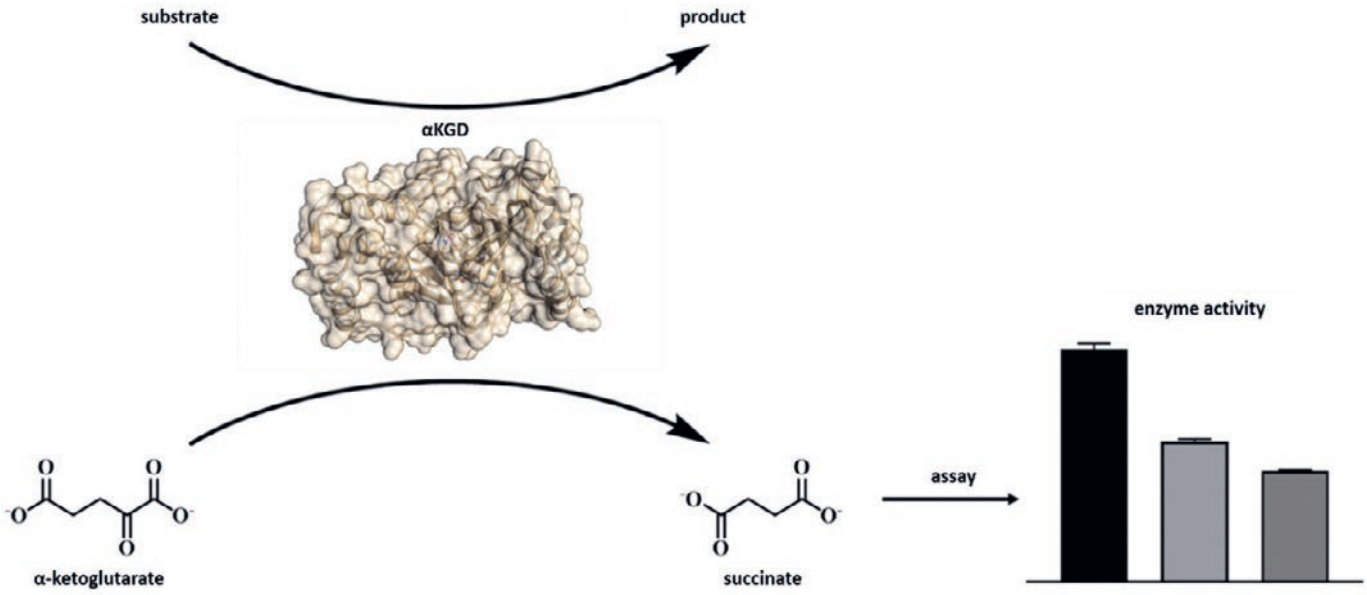


REACTION SCOPE: Overview of the reaction scope of αKGDs. The colour of the arrows indicates the substrates for the respective reactions. Figure adapted from Islam et al [3].

The co-substrate α-ketoglutarate is transformed to succinic acid in equimolar amounts to the main reaction product and the reaction can therefore be used to monitor the enzyme activity. In this way,



MECHANISM: Consensus mechanism of αKG-dependent hydroxylases. Mechanism and figure adapted from Islam et al. [3], Peters et al. [4] and Voss et al. [5].

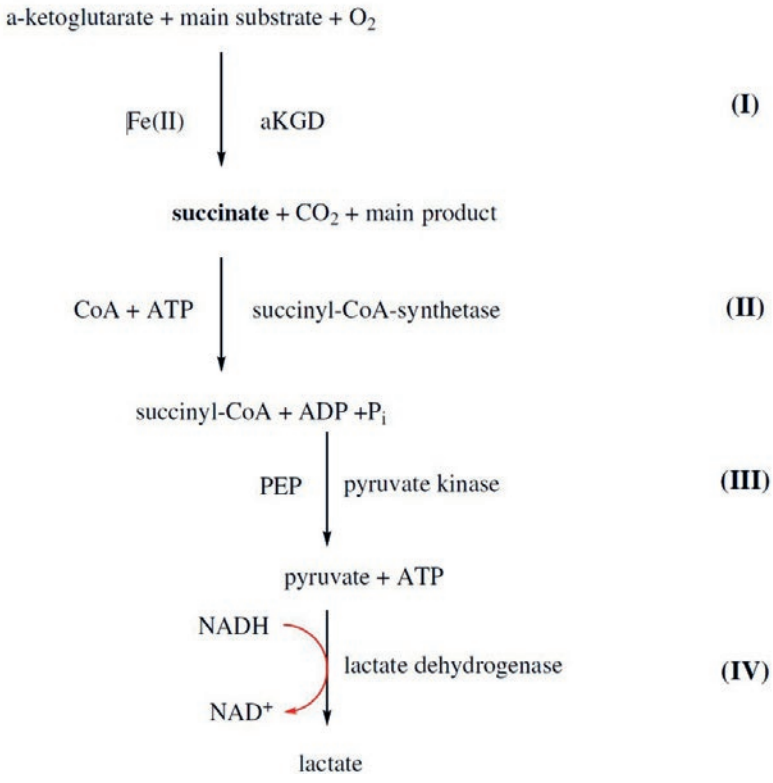


PRINCIPLE: Schematic representation of a universal screen for αKGDs. Crystal structure from Koketsu et al [6].

the activity measurement of various αKGDs can be carried out independently of their main reaction. In this thesis, the concept of two different approaches for the measurement of the side reactions was investigated and their use for the activity determination of a set of representative αKGDs was validated.

The first approach was designed to directly detect succinate formation by liquid chromatography coupled to mass spectrometry (LC-MS), while the second method was based on an enzymatic reaction cascade which allowed to detect succinate formation photometrically.

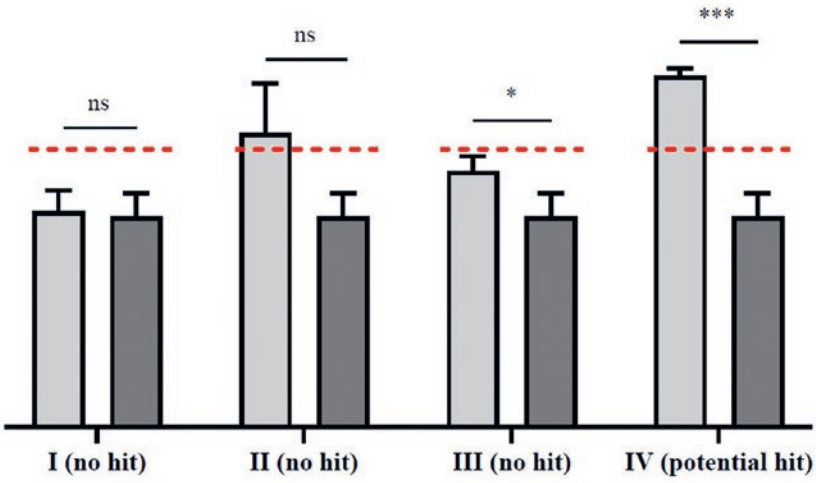
A library of 36 diverse αKGDs was modified by adding a N-terminal his-tag for enzyme purification. Soluble expression for 29 of 36 αKGDs was confirmed by SDS-PAGE. Based on the expression and substrate availability, four enzymes were selected and cultivated in batches. Initial validation of the LC-MS method and enzymatic assay was done by analysis of the biotransformation with the native substrates. For the identi-



ENZYME: Enzymatic assay to determine the activity of αKGDs. The biocatalytic transformation of the main substrate by αKGDs leads intrinsically to the formation of succinate (I). Succinate can be converted to succinyl-CoA by the enzyme succinyl-CoA-synthetase. Thereby, ATP is transformed to ADP (II). In a next step, pyruvate kinase transfers a phosphate group from phosphoenolpyruvate (PEP) to the formed ADP, under the release of an equimolar amount of pyruvate (III). In the last step, the formed pyruvate is reduced by lactate dehydrogenase. Thereby, NADH gets oxidized to NAD+ (IV). The decrease of NADH (indicated with a red arrow) can be measured at 340 nm. Scheme adapted from Luo et al. [7].

fication of potentially active reactions, different statistical tests were conducted. Both methods were able to detect significant formation of succinic acid and thus confirming enzyme activity of the enzymes towards their native substrates. Furthermore, both methods revealed unknown enzyme-substrate pairs as potentially active.

It is anticipated that with the conceptional validation of two methods capable of measuring αKGD activity independently from the enzyme's main reaction, the screening of diverse αKGDs libraries can be accelerated. By optimization of the screening parameters, such as the LC-MS method and conditions of the enzymatic assay, the economics of the screen could be further improved. Combining the here-established high-throughput screening with in silico approaches (virtual screening) is expected to further advance the search for industrially valuable C-H activation catalysts.



STATISTICS: Illustration of the analysis parameters to determine the significance of the difference between succinic acid concentration in biocatalysis (light grey) and uncoupled reactions (dark grey). The significance was determined by one-way ANOVA (enzymatic assay) or t-test (LC-MS), and is indicated above the bars. Additionally, a threshold was added, representing the limit of detection (LOD) and is indicated as a red line. In case I, neither of the parameters is full-filled, the biocatalytic reaction would be considered as not active. Case II shows a scenario, in which the threshold is surpassed, but the non-significance of the biocatalysis result compared to the negative control leads to a classification as "no hit". In case III, the succinic acid formation in the biocatalysis is significantly different from the uncoupled reaction. However, the LOD is not exceeded, which would also lead to a negative classification. Case IV shows a biocatalysis reaction, which would be considered as a hit. Both parameters are satisfied.

References

[1] J. J. Hutton, A. L. Tappel, S. Udenfriend, Cofactor and substrate requirements of collagen proline hydroxylase, Archives of Biochemistry and Biophysics, 1967, 118, 231–240

[2] C. J. Schofield, Z. Zhang, Structural and mechanistic studies on 2-oxoglutarate-dependent oxygenases and related enzymes, Current Opinion in Structural Biology, 1999, 9, 722–731

[3] M. S. Islam, T. M. Leissing, R. Chowdhury, R. J. Hopkinson, C. J. Schofield, 2-Oxoglutarate-Dependent Oxygenases, Annual Review of Biochemistry, 2018, 87, 585–620

[4] C. Peters, R. M. Buller, Industrial Application of 2-Oxoglutarate-Dependent Oxygenases, Catalysts, 2019, 9

[5] M. Voss, S. Honda Malca, R. Buller, Exploring the Biocatalytic Potential of Fe/_-Ketoglutarate-Dependent Halogenases, Chemistry – A European Journal, 2020, 26, 7336–7345

[6] K. Koketsu, Y. Shomura, K. Moriwaki, M. Hayashi, S. Mitsuhashi, R. Hara, K. Kino, Y. Higuchi, Refined Regio- and Stereoselective Hydroxylation of l-Pipecolic Acid by Protein Engineering of l-Proline cis-4-Hydroxylase Based on the X-ray Crystal Structure, ACS Synthetic Biology, 2015, 4, 383–392

[7] L. Luo, M. B. Pappalardi, P. J. Tummino, R. A. Copeland, M. E. Fraser, P. K. Grzyska, R. P. Hausinger, An assay for Fe(II)/2-oxoglutarate-dependent dioxygenases by enzyme-coupled detection of succinate formation, Analytical Biochemistry, 2006, 353, 69–74

Source: Jan Taubitz