Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Molecular Catalysis B: Enzymatic 39 (2006) 171–178
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Exploring the cupin-type metal-coordinating signature of acetylacetone dioxygenase Dke1 with site-directed mutagenesis: Catalytic reaction profile and Fe^{2+} binding stability of Glu-69 → Gln mutant

Grit D. Straganz [a,*], Sigrid Egger [a], Gianluca Aquino [c], Sabato D'Auria [c], Bernd Nidetzky [a,b,**]

[a] *Institute of Biotechnology and Biochemical Engineering, Graz University of Technology, Petersgasse 12, A-8010 Graz, Austria*
[b] *Research Centre Applied Biocatalysis, Graz University of Technology, Petersgasse 12, A-8010 Graz, Austria*
[c] *Institute of Protein Biochemistry, National Research Council C.N.R., Via Pietro Castellino 111, 80131 Naples, Italy*

Available online 28 February 2006

Abstract

Glu-69 belongs to a proposed active-site consensus motif His^{62}-X-His^{64}-X_4-Glu^{69} (where X is any amino acid) that acetylacetone dioxygenase Dke1 from *Acinetobacter johnsonii* shares with structurally related non-heme metal enzymes of the cupin protein superfamily. We report functional consequences of the site-directed replacement Glu-69 → Gln based on a detailed biochemical and kinetic characterization of the purified Dke1 mutant. Perturbations of the free energy profile of the wild-type caused by the mutation were surprisingly small, with key points of the reaction pathway such as β-diketone substrate binding, the rate-limiting reduction of dioxygen, and C–C bond cleavage essentially left unaltered. Release of Fe^{2+} from the mutant active site occurred at twice the wild-type rate, and the thermal stability of β-sheet secondary structure in Fe^{2+}-depleted apo-proteins was lower in the mutant. The substitution Glu-69 → Gln is thus remarkably silent regarding Dke1 function. These results do not support a unified catalytic or metal-coordinating role of Glu-69 (and its positional homologues) in O_2-dependent cupin-fold enzymes.

Keywords: Non-heme iron; C–C-bond cleavage; β-Diketone; Cupin; Metalloenzyme

1. Introduction

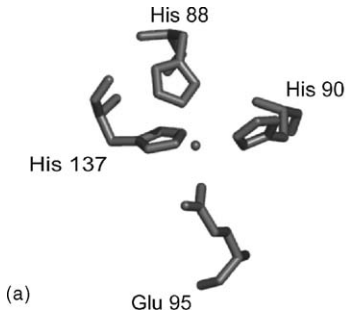
The enzymatic activation of dioxygen is crucial for all aerobic life in nature. Mononuclear non-heme metal active sites master the intricate chemistry of dioxygen to bring about a wide range of substrate transformations, which include the cleavage of C–C single and double bonds; hydroxylation; decarboxylation; and C–C bond formation [1]. High-resolution structural studies have revealed a common theme of non-heme metal coordination in enzymes that are unrelated by sequence and differ in three-dimensional fold. Two types of non-heme metal sites are found, apparently as a result of convergent evolution. The type 1 site is composed of two histidine and one carboxylate ligands [2]. The type 2 site contains three coordinating histidine residues whereby an additional carboxylate has a strongly conserved position close to the metal but not strictly coordinating it [3–5] (Fig. 1a).

The known, structurally characterized type 2 non-heme metal enzymes have a β-barrel (or "jelly roll") fold and belong to the cupin protein superfamily. Two consensus motifs have been proposed, which provide the signature for cupin metalloproteins (Fig. 1b): G-X-**H**-X-**H**-$X_{3,4}$-**E**-X_6-G and G-$(X)_5$-P-X-G-$(X)_2$-**H**-$(X)_3$-N where X is any amino acid and the key residues in the metal's first coordination shell are highlighted in bold. Dijkstra and co-workers have determined structure–function relationships for the side chains of the cupin signature based on their thorough studies of a fungal Cu^{2+}-dependent quercetin dioxygenase. The histidine residues are coordinating the metal cofactor, and the glutamate was assigned multiple probable functions: acid–base catalysis; proton relay; and modulator of the redox potential of the metal [14].

The β-diketone-cleaving enzyme Dke1 (EC 1.13.11.50) is a non-heme Fe^{2+}-dependent dioxygenase from *Acinetobacter johnsonii* [12,13]. It catalyzes the O_2-dependent conversion of

* Corresponding author. Tel.: +43 316 873 8414; fax: +43 316 873 8434.
** Corresponding author. Tel.: +43 316 873 8400; fax: +43 316 873 8434.
E-mail addresses: grit.straganz@tugraz.at (G.D. Straganz), bernd.nidetzky@tugraz.at (B. Nidetzky).

1381-1177/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2006.01.019



(b)

GER	--TNPP**H**I**H**PRAT**E**IGMVMKG GETFVIPRGLM**H**FQFN
ODC	GAIREL**H**W**H**KNA-**E**WAYVLKG GDLWYFPPGIP**H**SLQA
GDO	GEVAPT**H**R**H**SQSALRFVVD-G GDFVITPPWAW**H**DHVN
CDO	NKETTV**H**D**H**GQSIGCAMVLEG GECLISTKGLI**H**KMSN
ARD	EKFLNE**H**T**H**GE-D**E**VRFFVEG NDLISVPAHTP**H**WFDM
QDN	ALGVLP**H**I**H**QKHY**E**NFYCNKG GDYGSVPRNVT**H**TFQI
DKE	GSSFAS**H**I**H**AGPG**E**Y-FLTKG PSYGFESSGAL**H**GKTF
Consensus	G **H H** **E** G GD P G **H** N

Fig. 1. Typical active-site organisation and two consensus motifs for O_2-dependent metalloenzymes of the cupin protein superfamily. Panel (a): type 2 metal coordination by three histidine residues with a glutamate residue juxtaposed, exemplified by the active site of germin (Mn^{2+}-dependent oxalate oxidase) [6] and Panel (b): a multiple sequence alignment to underpin the conserved motifs in O_2-dependent cupin metalloenzymes. GER, oxalate oxidase [6]; ODC, oxalate decarboxylase [7]; GDO, gentisate dioxygenase [8]; CDO, cysteine dioxygenase [9]; ARD, acireductone dioxygenase [10]; QDN, quercetin dioxygenase [11]; DKE, diketone cleaving enzyme Dke1 [12,13], whereby for GDO and CDO no structural data are available. Note that the glutamate homologous to Glu-69 of Dke1 is conserved in all reported structures of type 2 oxygenases, while sequence alignments suggest an 'only' ∼70% conservation.

acetylacetone (2,4-pentanedione; PD) into methylglyoxal and acetate (Scheme 1).

Dke1 has a broad substrate specificity. It can accept β-diketones that represent a wide variety of substituents at both the central C-3 and the methyl groups of the natural PD substrate. Oxidative C–C bond cleavage in β-diketones proceeds with stoichiometric consumption of O_2, and one atom from dioxygen is incorporated into each reaction product upon the enzymatic bond fission. The primary structure of Dke1 clearly contains the cupin signature, with limited diversity noted in the second consensus motif (Fig. 1b). His-62, His-64 and His-104 are thus the putative ligands to Fe^{2+}. The X-ray crystal structure of an inactive form of Dke1 in which Fe^{2+} was replaced by adventitious Zn^{2+} supports a role of the three histidine residues in metal coordination [17]. The residue Glu-69, unlike its positional homologues in other cupin metalloenzymes, wholly points away from the metal cofactor in the Zn^{2+} structure, raising the question of what its function in native Dke1 could be.

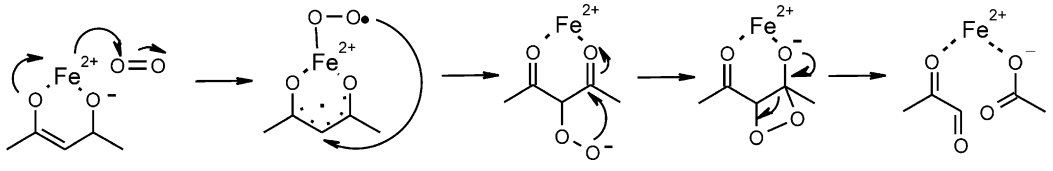
The proposed catalytic pathway of oxidative C–C bond cleavage is summarized in Scheme 1. The β-diketone substrate binds to the active site where it forms a metal-to-ligand charge transfer (MLCT) complex with Fe^{2+}. β-Dicarbonyls that are of physiological relevance generally have a pK_a of ∼9 and are likely to enter the active site in a charge neutral form [16]. Loss of a proton upon coordination as a monoanionic *cis*-β-keto-enolate to Fe^{2+} may require the participation of protein side chains, arguably that of Glu-69. The chemical transformation of the Fe^{2+}-bound substrate into an alkyl peroxidate intermediate is the rate-limiting step of the reaction catalyzed by the wild-type [16]. In this critical conversion, the catalytic reduction of dioxygen is performed. Finally, the peroxidate carries out intramolecular nucleophilic attack on the juxtaposed carbonyl groups, preferring the most electron-deficient of the two, to generate a dioxetane which then decomposes into the products. Glu-69 could take part in oxygen chemistry or the ensuing bond cleavage.

In this paper, site-directed mutagenesis was used to replace the original side chain of Glu-69 with the side chain of Gln, and the functional consequences in a purified Dke1 point mutant were analyzed in detail at different points of the reaction coordinate, as outlined in Scheme 1 and using methods previously established with the wild-type enzyme [15,16]. The results for Dke1 reveal that the positionally conserved Glu-69 is not always essential for enzyme function in cupin-fold type 2 metalloenzymes.

2. Material and methods

2.1. Materials

2,4-Pentanedione (PD), 1,1,1-trifluoro-PD (TFPD), 4,4-difluoro-1-phenyl-1,3-butanedione (DFPB), 4,4,4-trifluoro-1-phenyl-1,3-butanedione (TFPB), phenyl-1,3-butanedione (PB) and other chemicals were from Sigma Aldrich (St. Louis, MO, U.S.A.) except 1,1-difluoro-2,4-pentanedione (DFPD) which was from Matrix Scientific (Columbia, SC, U.S.A.). All chemicals were of the highest available quality and unless otherwise mentioned greater than 97% pure. *Pfu* DNA polymerase was from Promega (Madison WI, U.S.A.). *Dpn*I was obtained from MBI Fermentas (St. Leon-Rot, Germany). The other enzymes for molecular biology and the expression vector pKYB1 were from New England Biolabs (Beverly, MA, U.S.A.).



Scheme 1. Proposed reaction mechanism for Dke 1. The scheme is based on results of prior studies of the wild-type enzyme [13,15,16].