Acetylacetone-cleaving enzyme Dke1: a novel C–C-bond-cleaving enzyme from *Acinetobacter johnsonii*

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The toxicity of acetylacetone has been demonstrated in various studies. Little is known, however, about metabolic pathways for its detoxification or mineralization. Data presented here describe for the first time the microbial degradation of acetylacetone and the characterization of a novel enzyme that initiates the metabolic pathway. From an *Acinetobacter johnsonii* strain that grew with acetylacetone as the sole carbon source, an inducible acetylacetone-cleaving enzyme was purified to homogeneity. The corresponding gene, coding for a 153 amino acid sequence that does not show any significant relationship to other known protein sequences, was cloned and overexpressed in *Escherichia coli* and gave high yields of active enzyme. The enzyme cleaves acetylacetone to equimolar amounts of methylglyoxal and acetate, consuming one equivalent of molecular oxygen. No exogenous cofactor is required, but Fe^{2+} is bound to the active protein and essential for its catalytic activity. The enzyme has a high affinity for acetylacetone with a K_m of 9.1 μM and a k_{cat} of 8.5 s^{-1}. A metabolic pathway for acetylacetone degradation and the putative relationship of this novel enzyme to previously described dioxygenases are discussed.

Key words: acetylacetone degradation pathway, dicarbonyl cleavage, diketone cleavage, iron cofactor, oxygenase.

INTRODUCTION

Acetylacetone [2,4-pentanedione; Chemical Abstracts Service (CAS) no. 123-54-6] is a widely used industrial chemical with toxic side effects, as has been demonstrated in various studies. These include central neurotoxicity and possible effects on the immune system of mammals [1], as well as toxicity towards various aquatic organisms [2] and micro-organisms [3]. Limited studies indicate acetylacetone to be biodegradable [4]. The actual mechanisms of its decomposition in the environment have, however, not been investigated and a microbial route for acetylacetone detoxification or mineralization has not been reported. At present two enzymes are known to be capable of degrading acetylacetone [5,6]. They do this by hydrolytically cleaving the central C–C bond of the β-diketone moiety. These β-diketone hydrolases (EC 3.7.1.10) were isolated from poly(vinyl alcohol)-utilizing *Pseudomonas* strains and contribute to poly-(vinyl alcohol) degradation by hydrolysing the oxidized poly(vinyl alcohol) polymer. The hydrolases show relaxed substrate specificity towards uncharged β-diketones, among them acetylacetone. The physiological significance of these β-diketone hydrolases regarding acetylacetone degradation *in vivo* is, however, not well established.

In search of micro-organisms with the potential to mineralize acetylacetone, in order to study the underlying pathway and the enzyme machinery involved therein, we isolated an *Acinetobacter johnsonii* strain that grew on acetylacetone as the sole carbon source. Unexpectedly, it was found that the degradation of the growth substrate proceeds oxidatively via the initial oxygenative cleavage of a C–C bond of acetylacetone, followed by the further conversion of the cleavage products into non-toxic potential growth substrates. Here we describe the isolation, cloning and characterization of the initial oxidative C–C-bond-cleaving enzyme of the catabolic sequence.

EXPERIMENTAL

Chemicals and enzymes

Chemical compounds were purchased from Sigma Aldrich (St. Louis, MO, U.S.A.) at highest available purity, and octanedione was obtained from Lancaster Synthesis (Morecambe, Lancs., U.K.). Enzymes for molecular biological experiments were purchased from New England Biolabs (Beverley, MA, U.S.A.), if not otherwise stated.

Strain and media

A strain was isolated from sewage by growth at 30 °C in minimal medium M9 with acetylacetone (1 g · l^{-1}) as sole carbon source and was identified by 'Deutsche Sammlung von Mikro-organismen und Zellkulturen GmbH' as *Acinetobacter johnsonii* (DSMZ ID no. 98-849). The *A. johnsonii* strain was kept on minimal medium M9 plates, supplemented with acetylacetone as sole carbon source (1 g · l^{-1}). Expression constructs bearing *Escherichia coli* cells were kept on agar plates of Luria broth medium supplemented with 100 mg · l^{-1} ampicillin.

Protein production and purification

For the production of native enzyme by the wild-type *Acinetobacter* strain, a fermentation procedure described by Mandl [7] was applied. For the production of recombinant enzyme, *E. coli* BL21(DE3) (Stratagene, La Jolla, CA, U.S.A.) harbouring the plasmid expression vector containing the structural gene of the enzyme was grown in 100 mg · l^{-1} ampicillin-supplemented

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Luria broth medium at 30 °C to a D_{600} value of 0.6 on rotary shakers (125 rev./min) in baffled Erlenmeyer flasks. Cells were induced with 0.3 mM isopropyl β-D-thiogalactopyranoside and incubated for another 4 h. Cells were harvested by centrifugation (5000 g, 15 min, 4 °C), resuspended in 2 vol. of 50 mM potassium phosphate buffer, pH 7.5, and stored at -20 °C.

Cell lysates of induced cells were obtained by sonication according to standard procedures [7], or by two cycles in a French press (American Instruments Company, Silver Spring, MD, U.S.A.) at 8280 kPa. The supernatant was centrifuged at 100 000 g for 1 h at 4 °C. The acetylacetone-cleaving enzyme was purified from the cytosolic fraction by the following three or four step procedure (steps A–D, see below), using FPLC (Amersham Biosciences, Uppsala, Sweden). Buffers were equilibrated with nitrogen for 30 min to minimize the concentration of dissolved oxygen. It was crucial to perform purification at 4 °C and in the pH range of optimum stability, pH 7.5–8.0, to minimize activity losses.

Anion-exchange chromatography (step A)

The cytosolic fraction, containing 700–1000 mg of protein, was applied to a Sepharose Q Fast Flow column (30 ml; Amersham Biosciences). Unbound material was eluted with 20 mM Tris/HCl buffer, pH 7.5. A linear salt gradient (0–0.17 M NaCl over 2 column vol.) was applied, followed by the isocratic elution of active enzyme at 0.17 M NaCl.

Hydrophobic interaction chromatography (step B)

Active fractions from step A were pooled and concentrated 2–3-fold (Biomax 5; 5000 Da nominal molecular-mass cut-off; Centrifugal Filter Devices, Millipore, Bedford, MA, U.S.A.). Ionic strength of the sample was adjusted to 4 M NaCl before application to a Phenyl-Sepharose HP column (20 ml; Amersham Biosciences). After elution of unbound protein with 50 mM potassium phosphate buffer, pH 7.5, containing 4 M NaCl, acetylacetone-cleaving enzyme was detached with 1.2 M NaCl.

Gel filtration (step C)

The active fractions from step B were pooled and concentrated to 200 μl. The concentrate was applied to a Superdex 200 gel-filtration column (25 ml; Amersham Biosciences) and eluted with 50 mM potassium phosphate buffer, pH 7.5.

High-resolution anion-exchange chromatography (step D)

Optionally, when the purified protein from step C still showed impurities, it was applied to the high-resolution anion-exchange column ResourceQ (6 ml; Amersham Biosciences) and eluted with 20 mM Tris/HCl buffer, pH 7.5, with a gradient of 0–0.2 M NaCl over 20 column vol. The enzyme eluted at 0.17 M NaCl.

Protein determination and sequencing

Purified protein was tryptically digested and peptides were partially sequenced by electrospray ionization MS by PROTANA (Proteomics, Odense, Denmark). Protein content was determined by the BCA™ Method (Pierce, Rockford, IL, U.S.A.) by standard procedures. To determine the concentration of pure protein, UV absorbance of the denatured protein in 6 M guanidine/0.02 M K_2HPO_4, pH 6.5, at 280 nm was measured and protein concentration was determined with a theoretical ϵ value of 1.91 ($l \cdot g^{-1} \cdot cm^{-1}$) that was calculated based on sequence information.

Molecular mass and pI determination

The molecular mass of the denatured enzyme was measured by SDS/PAGE [8] according to standard procedures. The molecular mass was further determined by matrix-assisted laser-desorption ionization–time-of-flight MS (MALDI-TOF MS) at the Institute of Inorganic Chemistry, Graz University of Technology, Graz, Austria. The size of the native enzyme was estimated by gel filtration of 0.25 mg of purified protein with a Superdex 200 and a Superdex 75 column under standard conditions, with 50 mM potassium phosphate buffer, pH 7.5, and 0.0 or 0.15 M NaCl at a flow rate of 0.5 ml · min^{-1}. Calibration was performed using a Gel Filtration Standard Kit (Bio-Rad, Hercules, CA, U.S.A.) and BSA. Isoelectric focusing was done with the Ready Gel System and Isoelectric Focusing Calibration Kit (pI 4.45–9.6) from Bio-Rad according to the manufacturer's recommendations.

Cross-linking was performed by addition of 5 μl of aqueous solutions of glutaraldehyde (500–0.1 mM) to 20 μl of purified enzyme (0.3 mg/ml) in 50 mM K_2HPO_4, pH 7.5, and incubation on ice for 15 min. The reaction was stopped by addition of 1 μl of 1 M Tris buffer, pH 8.0. The samples were then denatured and analysed by SDS/PAGE and silver staining with a PhastSystem™ (Amersham Biosciences) according to the manufacturer's procedure.

UV photometric assays

If not otherwise stated, the enzyme activity was determined UV-spectrophotometrically (Spectronic Genesis 2PC; Thermo Spectronic, Rochester, NY, U.S.A.) by measuring the decrease in the respective diketone at 280 nm (acetylacetone, $\epsilon_{280} = 2240$ $l \cdot mol^{-1} \cdot cm^{-1}$; 2,4-octanedione, $\epsilon_{280} = 2000$ $l \cdot mol^{-1} \cdot cm^{-1}$; 3-methylpentanedione, $\epsilon_{280} = 580$ $l \cdot mol^{-1} \cdot cm^{-1}$; 2-acetylcyclohexanone, $\epsilon_{280} = 2400$ $l \cdot mol^{-1} \cdot cm^{-1}$; 3,5-heptanedione, $\epsilon_{280} = 1200$ $l \cdot mol^{-1} \cdot cm^{-1}$; determined at pH 7.5, 25 °C). The standard conditions for the activity assay were air-saturated 50 mM potassium phosphate buffer, pH 7.5, at 25 °C, 0.25 mM acetylacetone and 5–100 μl of appropriately diluted enzyme, in a total volume of 1 ml. The reaction rates were determined by using the microcomputer regression program provided by the spectrophotometer (Winspec) based on the first 10 absorbance measurements made at 5 s intervals. One unit of enzyme activity was defined as that which cleaved 1 μmol of acetylacetone/min under standard conditions. Apparent K_m and k_{cat} values were calculated from initial rate measurements with varying substrate concentrations (3 μM–2 mM) in air-saturated buffer under standard conditions, using Michaelis–Menten curve fit provided by Microcal OriginPro 6.1 (OriginLab, Northampton, MA, U.S.A.).

Oxygen measurements

Oxygen consumption during the enzyme reaction as well as cell respiration were measured with an oxygen electrode cell (Digital Oxygen System model 10; Rank Brothers, Cambridge, U.K.) as described previously [9]. The reaction mixture contained air-saturated 50 mM potassium phosphate buffer, pH 7.5, at 25 °C. Varying amounts of substrate to concentrations of 0.01–2 mM were added and the resulting decrease of oxygen was determined.

Inhibition and reactivation studies

Inhibition studies were conducted by adding purified enzyme (0.1 mM) to freshly prepared solutions of the respective inhibitors (1–2.5 mM) in 20 mM Tris buffer, pH 7.5, which was then

Table 1   Primers used in this study

Above the degenerate oligonucleotide primers the peptide sequences are given, on which the primerdesign was based. Underlined regions indicate engineered restriction sites.

PCR primer	Sequence
Peptide sequence P01	E Q E G G S T A Y A P S Y G F E 5′-GARCARGARGGVGGVTCDACMGCWTAYGCNCCWTCDTAYGGVTTYGA-3′
Peptide sequence P04	W A G Q A E A W 5′-CCANGCKCCYTGVGCYTCVGCYCA-3′
P05	5′-TCTTCAGGTGCATTGCATGG-3′
P06	5′-ACCATGCAATGCACCTGAAG-3′
PDkNd	5′-CTATA<u>CATATG</u>GATTATTGTAATAAAAAACACACTG-3′
PDkBam	5′-AT<u>GGATCC</u>TCAGGCAGCCTCATTTTTG-3′

incubated at room temperature. Relative activity was expressed as a percentage of the activity of the enzyme incubated in buffer without inhibitor. For reactivation studies, 100 μl of phenanthroline-inhibited enzyme was incubated in 1.9 ml of Tris buffer, pH 7.5, with an excess of the respective metal salts (2 mM); in the case of Fe^{2+}, ascorbate (2 mM) was added to prevent oxidation to Fe^{3+}. Activity was then measured in the oxygen electrode cell under standard conditions, by addition of 10 μl of the respectively treated enzyme solution to a total volume of 1 ml.

NMR measurements

The products of the enzymic reaction were measured directly in the reaction mixtures by *in situ* ^{1}H-NMR spectroscopy in 50 mM potassium phosphate buffer, pH 7.5 [10]. A Varian Gemini 2000 (200 MHz) and a Varian Unity 500 (500 MHz), both narrow-bore magnets and equipped with the respective 5 mm broadband probe heads, were used. The NMR tube was rotated at 20 rev./s. For a lock a ^{2}H$_2$O vortex capillary was added to avoid ^{1}H–^{2}H exchange reactions. The overwhelming water signal was suppressed with a presaturation method [11,12]. The following parameters were adjusted for ^{1}H frequencies of 200 and 500 MHz, respectively: presaturation duration of 1.0 s, ^{1}H pulse angle of 90°, an acquisition time of 2.0 s and a relaxation delay of 1.5 s. A total of 64 scans were accumulated and after a zero filling to 32000 data points the free induction decay was Fourier transformed. The water signal (4.70 p.p.m.) was used as a reference. All experiments were performed in 50 mM potassium phosphate buffer, pH 7.5, with enzyme preparations that were free from Tris buffer, since methylglyoxal reacts with the tris-(hydroxymethyl)-aminomethane molecule, forming several by-products [13].

Cloning of the diketone-cleaving enzyme gene *dke1*

Total DNA from *A. johnsonii* was isolated with Qiagen Genomic-tips 500/G according to the manufacturer's procedure (Qiagen, Hilden, Germany). Amplification with degenerate primers (Table 1) was performed in a total volume of 50 μl, using 100 ng of chromosomal *A. johnsonii* DNA as a template, 400 ng of primers, 5 μl of dNTPs (1 mM each), 5 μl of Qiagen PCR buffer and 1 unit of Qiagen HotStar *Taq* DNA polymerase, which was activated at 95 °C for 15 min. The PCR regime applied was 10 cycles at 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min, followed by 20 cycles at 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min. The resulting PCR products were directly

cloned with the TOPO TA Cloning Kit as described by the manufacturer (Invitrogen, Carlsbad, CA, U.S.A.) and sequenced. The cloned 150 bp PCR fragment was labelled with digoxygenin according to the protocol of the Dig DNA Labelling & Detection Kit (Roche, Mannheim, Germany) and used for Southern blot hybridization as a probe with *Eco*RI-digested chromosomal DNA, according to a standard procedure (Boehringer Mannheim, Mannheim, Germany), giving a band of 3 kb. Consequently a gene library of 2–4 kb *Eco*RI fragments from *A. johnsonii* DNA in Bluescript SK II(−) (Stratagene) was constructed by standard techniques [14]. PCR reactions were performed using 100 ng of gene library plasmid as a template with combinations of the vector-specific high-temperature primers T3 20-mer and T7 22-mer (Stratagene), and gene-specific primers P05 and P06 (Table 1) as described previously.

Construction of the expression clone

Primers were designed based on the sequence information gained from the 1 and 2 kb PCR products, which had been amplified from the gene library. The forward primer PDkNd (Table 1) was engineered with an *Nde*I site, which overlapped the initiation codon of *dke1*. The reverse primer PDkBam (Table 1), located downstream of the stop codon, was designed with a *Bam*HI site. The *dke1* gene was amplified from *A. johnsonii* genomic DNA. Amplification was performed in a total volume of 100 μl with 2 units of *Pwo* DNA polymerase (Roche), which has proofreading activity, 50 ng of chromosomal DNA as a template, 200 ng of each primer, 10 μl of dNTPs (1 mM each), 10 μl of *Pwo* PCR buffer, applying 30 cycles at 94 °C (1 min), 57 °C (1 min) and 72 °C (2 min). The resulting PCR product was cloned into the *Nde*I/*Bam*HI site of pTXB1 (New England Biolabs), thus allowing inducible expression of recombinant Dke1 (rDke1) from the strong T7 promoter.

Sequence analysis

Blast and PSI Blast searches were performed at the National Centre of Biotechnology Information (NCBI) [15]. Motif searching was done with various programs available at http://www.expasy.ch, e.g. by Prosite and Pfam at Washington University, St. Louis, MO, U.S.A.

Metal analysis

Total reflection X-ray fluorescence analysis was performed on an EXTRA IIA (Atomica Instruments) at the Institute of Inorganic

and Analytical Chemistry, Johann Wolfgang Goethe University, Frankfurt, Germany. Fe^{2+} and Zn^{2+} were also determined spectrophotometrically. Fe^{2+} was measured by the *o*-phenanthroline method [16] in 20 mM Tris/HCl buffer, pH 7.5. To determine total iron, ascorbate (2 mM) was added to reduce Fe^{3+} to Fe^{2+}. Zn^{2+} was determined by the bicolorimetric dithizone method [17].

RESULTS

Isolation of a novel acetylacetone-cleaving enzyme

A bacterial strain was isolated from sewage by enrichment cultures on minimal medium, which contained acetylacetone as the sole source of carbon. The strain was identified as *A. johnsonii* (DSMZ ID no. 98-849). Cell extracts of acetylacetone-grown cells showed the conversion of acetylacetone into acetate, lactate and pyruvate. The primary enzyme of the metabolic sequence was purified as summarized in Table 2 and Figure 1. Despite the pronounced activity losses during hydrophobic-interaction chromatography, we had to include this step to get pure protein (Figure 1). Some minor impurities of higher molecular mass were detached by gel filtration. A single protein species that degraded acetylacetone was obtained (Table 2). This acetylacetone-cleaving enzyme from *A. johnsonii* is further on designated as native Dke1 (nDke1).

Enzyme properties

With SDS/PAGE under reducing as well as non-reducing conditions and by isoelectric focusing, a single protein band with an apparent molecular mass of approx. 16.6 kDa (Figure 1a) and an isoelectric point of 4.4 was found. The molecular mass of the purified enzyme, determined by MALDI-TOF MS, was 16607 Da. Size-exclusion chromatography showed a single peak, revealing a molecular mass of approx. 65 kDa for the native enzyme. Furthermore, when purified protein that had been treated with an excess of the cross-linking reagent glutaraldehyde was submitted to SDS/PAGE, a band at approx. 70 kDa formed, concomitant with the disappearance of the monomer band at 16.6 kDa. This indicates that the subunits of the enzyme had been linked covalently. Consistent molecular masses of 65–70 kDa found by gel filtration as well as by cross-linking (Figure 1b) strongly suggest that nDke1 exists as a functional tetramer in solution. These findings are also in agreement with dynamic-light-scattering measurements of purified Dke1 (results not shown). The UV–visible absorbance spectrum of nDke1 showed a single symmetrical peak at 280 nm, but no other bands or distortions that might indicate the presence of non-protein prosthetic groups.
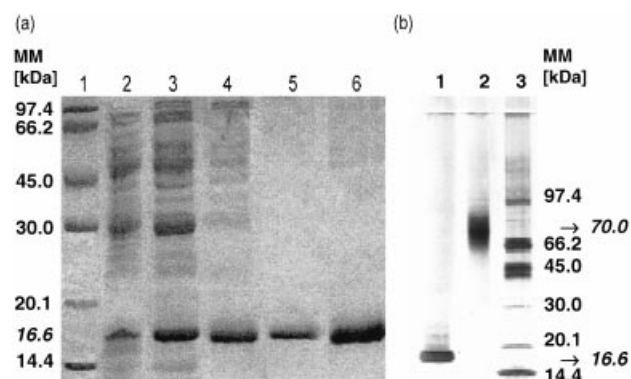

Figure 1   Purification and properties of the wild-type protein nDke1

(**a**) The active fractions obtained during the purification procedure of nDke1 were submitted to SDS/PAGE (12.5% gel) under reducing conditions and visualized with Coomassie Blue stain. Lane 1, molecular-mass standards, with the molecular masses (MM) of the respective bands shown on the left. The estimated mass of Dke1, 16.6 kDa, is indicated in italic; lane 2, cell extract (40 μg of protein); lane 3, pooled active fractions after the anion-exchange column (50 μg of protein); lane 4, active fractions after hydrophobic-interaction chromatography (20 μg of protein); lane 5, purified protein after gel filtration (5 μg of protein); lane 6, purified protein (25 μg of protein). (**b**) Cross-linking studies with the purified protein (18 μM) were performed as described in the text. The resulting samples were visualized on an 8–15% SDS gradient gel with silver staining. Lane 1, purified protein (0.3 μg); lane 2, purified protein, treated with an excess of glutaraldehyde (10 mM); lane 3, molecular-mass standards (indicated on the right).

Physiological significance of Dke1

In order to investigate the physiological significance of Dke1, its *in vivo* activity was studied. Growth of the *A. johnsonii* strain on acetylacetone in minimal medium was an order of magnitude slower than that achieved with various other carbon sources, such as acetate, lactate and pyruvate. An excess of acetylacetone (> 15 mM) completely inhibited growth. On addition of acetylacetone to cell suspensions of acetylacetone-grown cells, a 10-fold increase in cell respiration rates was found, giving 250 μM $\cdot$ min^{-1} $\cdot$ D_{600} unit^{-1}. Similar acetylacetone-depletion rates, determined spectrophotometrically, were observed. More than 95% of the enzymic activity of acetylacetone-grown cells was retained in the soluble 100000 *g* fraction of the resulting lysates, giving evidence that nDke1 is a cytosolic enzyme that is truly functional *in vivo*. A rough estimate based on the specific activity of fully active pure enzyme (to be shown later) is that Dke1 constitutes approx. 2% of total soluble protein in the cell extract of acetylacetone-grown cells. In marked contrast, with cells that had been grown on other carbon sources lacking acetylacetone (acetate, lactate, pyruvate, Luria broth), no oxygen consumption additional to the basal respiration rates

Table 2   Purification of nDke1

The purification scheme shows a typical set of data found during the purification of nDke1. The first purification step involved anion-exchange chromatography on a Sepharose Q Fast Flow column (QFF), followed by hydrophobic-interaction chromatography (HIC), which included subsequent concentration by ultrafiltration, and gel-filtration.

Purification step	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Recovery (%)	Fold purification
Cell extract	24	1008	642	0.6	100	1.0
QFF	20	256	625	2.4	97	3.8
HIC	0.21	25	169	6.7	26	10
Gel filtration	3	13.5	134	9.9	21	16

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  1  TCAGCAAAAGATAGATCGTTTTTTTTTATGAAAGAAATCTATTGTTCTATATATTTTTGTAGTTCATTTTTAAGCAAACACTTGTGCGTTT   90
                                                                                dke1 →
 91  TTAGACAATTTTCCAAATCTCATTTCAATATTATGAAGATGTGTCATGTGTAGACACACATATAAGGAGATATGAAATGGATTATTGTAA  180
                                                                          M  D  Y  C  N
181  TAAAAAACACACTGCTGAAGAATATGTAAAAATTTCAGATAATAACTATGTTCCTTTCCCAGAAGCATTTTCTGATGGTGGAATCACTTG  270
      K  K  H  T  A  E  E  Y  V  K  I  S  D  N  N  Y  V  P  F  P  E  A  F  S  D  G  G  I  T  W
271  GCAATTATTACATTCGTCACCAGAACAAGTAGTTGGACGGCAATTTCAACTGTCCTGCTGGCTCATCTTTTGCTTCTCATATTCATGC  360
      Q  L  L  H  S  S  P  E  T  S  S  W  T  A  I  F  N  C  P  A  G  S  S  F  A  S  H  I  H  A
361  TGGCCCCGGTGAATATTTCCTGACTAAGGGAAAAATGGAAGTGCGTGGTGGCGAGCAAGAGGGTGGTAGCACTGCTTATGCACCAAGCTA  450
      G  P  G  E  Y  F  L  T  K  G  K  M  E  V  R  **G  G  E  Q  E  G  G  S  T  A  Y  A  P  S  Y**
451  CGGTTTTGAATCTTCAGGTGCATTGCATGGTAAAACTTTCTTTCCTGTCGAAAGCCAGTTCTATATGACCTTTTTAGGGCCGCTTAATTT  540
      **G  F  E  S  S  G  A  L  H  G  K**  T  F  F  P  V  E  S  Q  F  Y  M  T  F  **L  G  P  L  N  F**
541  TATTGATGATAACGGAAAAGTTATTGCATCGATTGGTTGGGCTGAAGCTCAAGGTGCATGGTTAGCTACCAAAAATGAGGCTGCCTGACT  630
      **I  D  D  N  G  K  V  I  A  S  I  G  W  A  E  A  Q  G  A  W  L  A  T**  K  N  E  A  A  *
     ORF2 →
631  ATGTCATTAACTCTTGAAGTTTTTGATCATCACTTAGGTGCTTTTGCATTGGGTGTTGATGAGCTTTTAAAAGATTACGATGAATCTTCG  720
      M  S  L  T  L  E  V  F  D  H  H  L  G  A  F  A  L  G  V  D  E  L  L  K  D  Y  D  E  S  S
721  GTAATTTTCACTAATAGTGGACATTCGCGTGGATTGGAGGAAATCCGTACTTTCTTTAGTAACTTTCTTGACAGCCTACCTGATGATTTT  810
      V  I  F  T  N  S  G  H  S  R  G  L  E  E  I  R  T  F  F  S  N  F  L  D  S  L  P  D  D  F
811  TGGAATGATTTTCAGGTTTTGGAAAAAGAGGTGATGGCTGAAGTTGCTTATCTAGTCTGGAGCGCTAAGCCTTATGTGGCTCTGGCAACG  900
      W  N  D  F  Q  V  L  E  K  E  V  M  A  E  V  A  Y  L  V  W  S  A  K  P  Y  V  A  L  A  T
901  GATACCATGTTGATCCGTGACGGTAAAATTGTTACCCAGACATTTACCAAATTTTAAGTGTAAAAGGATATTCACATGAAACTTAAAGAT  990
      D  T  M  L  I  R  D  G  K  I  V  T  Q  T  F  T  K  F  *
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Figure 2   Sequences of the open reading frames and their flanking regions

The cloned DNA fragment contained two tandemly arranged open reading frames. The first open reading frame (*dke1*) coded for a 153-amino-acid protein and contained previously determined peptide sequence (bold letters). The stop codons are marked by asterisks. *dke1* is preceded by a putative promoter region. The promoter consensus sequence, which is highly similar to the classical *E. coli* promoter consensus sequence [18], is marked with a double underline. Potential Shine–Dalgarno sequences [19] are underlined. *dke1* is directly followed by ORF2, which codes for a putative protein consisting of 108 amino acids. The sequence was determined by sequencing both senses of the DNA strand.

$(150–200\ \mu\text{M} \cdot \text{min}^{-1} \cdot D_{600}\ \text{unit}^{-1})$ was observed on addition of acetylacetone, nor was any acetylacetone-cleavage activity observed in the resulting cell extracts. Taking into account the limits of the sensitive UV assay, when measuring the enzyme activity in cell extracts it can be estimated that the Dke1 level in acetylacetone-grown cells was at least 300-fold higher than the level in non-induced cells. This gives evidence that, directly or indirectly, acetylacetone has a dramatic inducing effect on the expression of functional nDke1 in *A. johnsonii*.

The gene for acetylacetone-cleaving enzyme Dke1

Partial sequencing of tryptically digested protein gave three peptide sequences: (i) V(I/L)AS(I/L)GWAEAQGAW(I/L)-ATK, (ii) (I/L)GP(I/L)NF(I/L)DDNGK and (iii) GGEQEG-GSTAYAPSYGFESSGA(I/L)HGK. Based on this partial peptide sequence information, the respective DNA regions were cloned and sequenced as described in the Experimental section. Sequence analysis showed two open reading frames, which were tandemly arranged (Figure 2). The first open reading frame, which codes for a 153-amino-acid protein, was designated as *dke1*, since it contained the previously determined peptide sequences of Dke1. The calculated molecular mass of the deduced amino acid sequence of 16607 kDa exactly matches the value found by MALDI-TOF MS. *dke1* is preceded by a putative promoter region, which resembles the classic conserved promoter consensus sequence defined for *E. coli* [18]; Shine–Dalgarno consensus sequences precede both open reading frames [19]. The open reading frame ORF2 codes for a putative protein consisting of 108 amino acids. *dke1* was overexpressed in *E. coli* BL21(DE3), yielding active rDke1, with a specific activity of 1.9 units/mg of soluble protein in cell extracts.

Although several microbial genomes are already totally sequenced, Blast and PSI Blast searches [15] at the NCBI showed no significant similarities of Dke1 or ORF2 to annotated protein sequences. No motifs or consensus patterns indicative of a certain enzyme class could be identified by computational methods.
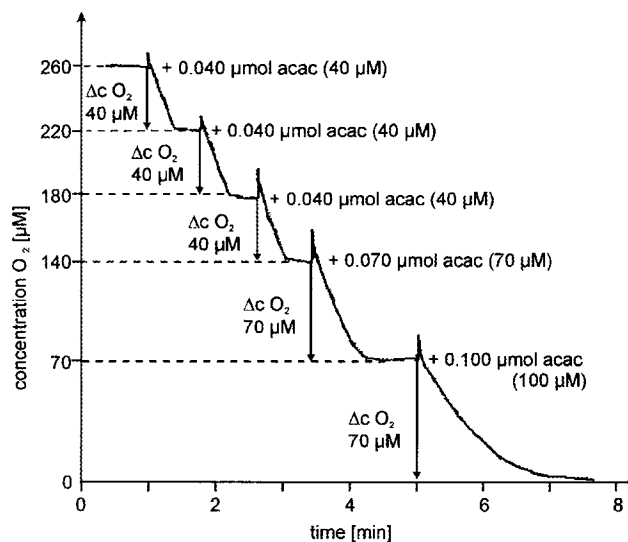

Figure 3   Oxygen consumption in Dke1-catalysed acetylacetone cleavage

Defined amounts of acetylacetone (acac) were added stepwise to an air-saturated (260 μM O$_2$) solution of rDke1 (0.1 unit) in 50 mM potassium phosphate buffer, pH 7.5, at 25 °C, in a total volume of 1 ml, leading to acetylacetone concentrations of 40, 70 and 100 μM. Oxygen consumption (Δc) was equimolar to the quantity of acetylacetone added. Final addition of acetylacetone in excess led to a total consumption of oxygen.

Acetylacetone-cleavage reaction

A stoichiometric decrease of molecular oxygen during the enzyme reaction was observed by polarographic methods. One μmol of O$_2$ was consumed per μmol of acetylacetone added to the reaction mixture until all oxygen was depleted (Figure 3). No H$_2$O$_2$ was formed during acetylacetone cleavage, as was proven by addition of catalase to the reaction mixture and by the sensitive horseradish peroxidase assay [20].
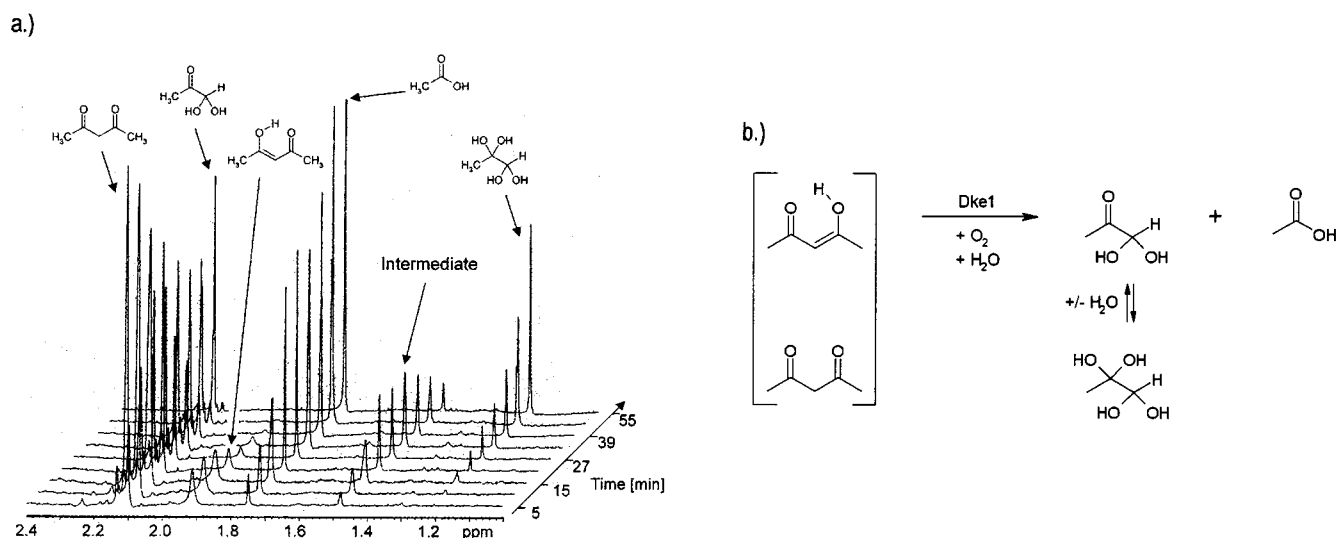
a.)

b.)

Figure 4   *In situ* ^{1}H-NMR of Dke1-catalysed cleavage of acetylacetone to methylglyoxal and acetate

Starting conditions were 5.0 mM acetylacetone in 50 mM potassium phosphate buffer at 25 °C and 40 μg of rDke1 in a total volume of 10 ml. Samples were aerated between each measurement. (**a**) Stack plot of the spectra taken every 5–10 min. (**b**) Reaction scheme of the Dke1-catalysed cleavage of acetylacetone and subsequent hydratation reactions, deduced from ^{1}H-NMR and oxygen-consumption measurements. Acetylacetone, which is present in its diketo and enol forms, is cleaved to methylglyoxal and acetate. Methylglyoxal is initially found as a monohydrate, which is then partially converted into the dihydrate.

Table 3   Correlation between Fe^{2+} content and enzyme activity of purified Dke1

Three different preparations each of purified nDke1 and rDke1 were investigated spectrophotometrically for their activity and metal content. Means $\pm$ S.D. regarding the iron content and specific activities of the respective three preparations are shown. In a duplicate assay, enzyme (4 mg of protein in 200 μl) from one active enzyme preparation (preparation 3) was deactivated by addition of H$_2$O$_2$ (2.5 mM) and subsequently submitted to gel filtration, giving preparations 3′ and 3″.

Preparation	Iron (zinc)/subunit	Specific activity (units/mg of protein)	Activity/enzyme-bound iron (units/μmol of Fe^{2+})
1–3, nDke1	0.4 $\pm$ 0.2 (0.0–0.4)	11 $\pm$ 5	457 $\pm$ 50
4–6, rDke1	0.94 $\pm$ 0.1 (0.0)	28 $\pm$ 3	465 $\pm$ 48
3′ and 3″, nDke1 + H$_2$O$_2$	0.0 $\pm$ 0.0 (0.3)	0.0	–

The enzymic cleavage reaction was monitored by ^{1}H-NMR [10]. Pure native and recombinant Dke1 formed 1 mmol of acetate, δ_H 1.76 (3H, s, CH$_3$COOH)/mmol of acetylacetone, which is present in its diketo [δ_H 2.11 (6H, s, CH$_3$CO)] and enol form [δ_H 1.90 (6H, s, CH$_3$COH)] in aqueous solutions [21]. Concomitant with acetate, two signals emerged. Based on standard addition experiments they were assigned to the methyl-group-bound hydrogen of the two solution forms of methylglyoxal, its monohydrate {δ_H 2.14 [3H, s, CH$_3$COCH(OH)$_2$]} and its dihydrate {δ_H 1.28 [3H, s, CH$_3$C(OH)$_2$CH(OH)$_2$]} (Figure 4a [22]). The integrals over the ^{1}H signals of the methyl groups showed that both acetate and methylglyoxal were formed in amounts that were equimolar to acetylacetone cleaved. In the ^{1}H-NMR time course of the Dke1-catalysed reaction an as-yet unidentified singlet (δ_H 1.48) appeared (Figure 4a) that was also observed in aqueous solutions of acetylacetone and methylglyoxal, suggesting a condensation product of these two compounds. All results taken together, the reaction catalysed by Dke1 can be described as the consumption of 1 molecule of O$_2$

and the concomitant cleavage of the C–C bond of 1 molecule of acetylacetone, yielding equimolar amounts of acetate and methylglyoxal. In Figure 4(b) a reaction scheme is outlined that summarizes the findings from UV, oxygen-consumption measurements and the time course from ^{1}H-NMR.

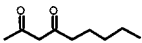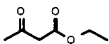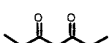The actual route of acetylacetone degradation by *A. johnsonii* was investigated further. Whole cells from *A. johnsonii* growing on acetylacetone consumed the substrate without the concomitant formation of any detectable organic cleavage products, whereas with cell lysates from *A. johnsonii* acetate, lactate and pyruvate were found as final products. Partially purified nDke1 catalysed the conversion of acetylacetone into equimolar amounts of acetate and lactate. On the contrary, with cell lysates of recombinant *E. coli* acetylacetone was cleaved to methylglyoxal and acetate.

Dke1 is an Fe^{2+}-dependent enzyme

The native enzyme did not show requirement for any exogenous cofactor. It had its highest activity between pH 6.5 and 8.5, with a sharp decrease above and below this pH range. Addition of metal-chelating agents such as EDTA, KCN and *o*-phenanthroline [23] lead to largely irreversible losses of enzyme activity. When using snapshot measurements at 10 min incubation time under the conditions described in the Experimental section, 64 % (EDTA) to 90 % (*o*-phenanthroline) activity had been lost. The latter compound formed a deep red colour, which indicated the presence of Fe^{2+} in the enzyme preparation. Activity of *o*-phenanthroline-inactivated enzyme recovered partially (> 30 %) by addition of Fe^{2+}, whereas no reactivation could be achieved with Fe^{3+} or with various other metal ions (Zn^{2+}, Mn^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+}). The enzyme was unstable in the presence of oxidizing reagents. Oxidizing K$_3$Fe(III)(CN)$_6$ (2.5 mM) decreased enzyme activity by 80 % within 10 min, whereas its reducing counterpart K$_4$Fe(II)(CN)$_6$ had no effect on enzyme activity. The addition of equimolar amounts of H$_2$O$_2$ (0.1 mM) even resulted in an immediate and total inhibition of enzyme

Table 4   Substrate spectrum of Dke1

Acceptance of the respective substrates was firstly tested with the Clark oxygen electrode. Apparent k_{cat} and k_m values were then determined by initial rate measurements either by spectrophotometrically recording the depletion of substrate or — in the case of ethylacetoacetate and 3-methyl-2,4-pentanedione — by measuring the rate of oxygen consumption with the Clark oxygen electrode. Values determined were within the experimental error of 20 %. Initial oxygen concentration in the reaction mixtures was 260 μM.

Structure	Compound	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} · mM^{-1})
	Acetylacetone	8	940
	3-Methyl-2,4-penatedione	12	40
	2,4-Octanedione	9	280
	2-Acetylcyclohexanone	12	210
	Ethylacetoacetate	10	95
	3,5-Heptanedione	6	750
	3-Oxobutanoate	0.00	0
	4-Hydroxy-4-methyl-2-pentanone	0.00	0

activity concomitant with the loss of enzyme-bound iron that could then be detached by gel filtration (Table 3).

Purified rDke1 reproducibly contained 0.9–1.0 iron atom/ subunit. Preparations of electrophoretically pure nDke1 showed various sub-stoichiometric amounts of iron and zinc (Table 3). The actual reason for the observed metal losses and for the presence of zinc in most nDke1 preparations remained unclear. Changes in the purification procedure are one possible reason for the differences between nDke1 and rDke1 preparations with respect to their metal content. When rDke1 was purified, the rough hydrophobic-interaction chromatography step, which had caused substantial losses of activity during the purification of nDke1, was left out. Remarkably, even in partially inactivated preparations containing zinc, a clear correlation of enzymic activity and Fe^{2+} content was observed (Table 3). A constant ratio of acetylacetone-cleavage rate versus iron content of 410–520 units/μmol of Fe^{2+} was found throughout all analysed enzyme preparations, whereas zinc obviously did not contribute to enzyme activity. All in all, irrespective of the extent of metal loss in various enzyme preparations, the enzyme activity based on the molar content of Fe^{2+} per subunit was invariant. Therefore, this provides good evidence that one ion of iron is incorporated into one subunit of the fully active enzyme.

Substrate specificity of Dke1
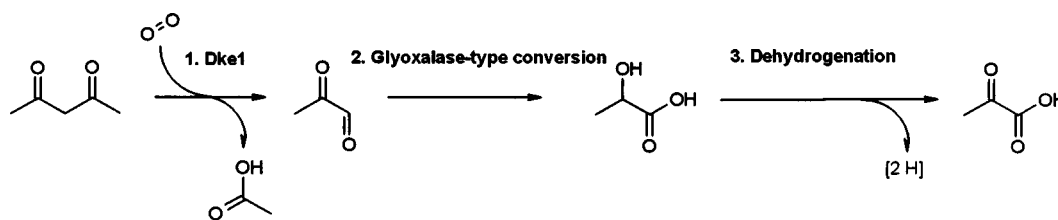
Kinetic parameters for the acetylacetone-cleavage reaction were determined from initial rate measurements in which the depletion of acetylacetone was monitored. The apparent turnover number based on molar concentrations of Fe^{2+} was 8.5 ± 0.8 s^{-1} and the apparent K_m for acetylacetone was 9.1 ± 1.5 μM. Screening for alternative substrates of Dke1 was carried out and various compounds structurally resembling acetylacetone were tested

under standard reaction conditions. Structures showing clear activity were selected for more detailed characterization. Table 4 provides a summary of the substrate specificity of Dke1. The enzyme is not absolutely specific for acetylacetone, given that related β-dicarbonyl compounds are converted with robust activity. However, k_{cat}/K_m values show clearly that, although substitutions at the carbon adjacent to the reactive β-dicarbonyl groups are tolerated, changes in non-reactive substrate parts lead to substantial changes in specificity constants. Interestingly, in spite of 50-fold variations in second-order rate constants over the range of substrates, k_{cat} values are hardly affected in response to changes in substrate structure. A possible interpretation is that β-dicarbonyl cleavage by Dke1 is controlled by a common rate-limiting step in all substrates. Compounds not converted by Dke1 provide interesting clues of structure requirements for enzyme activity. 4-Hydroxy-4-methyl-2-pentanone, which lacks a β-dicarbonyl structure, is not a substrate of Dke1. Remarkably, contrary to its ester analogue, 3-oxobutanoate is not accepted, what might be due to its charged carboxylate moiety. Several compounds that are known as substrates for some C–C-bond-cleaving oxygenases, such as various catechols, quercetin, 2,4′-dihydroxyacetophenone or ascorbate, were tested, but none of them was converted within the limits of detection of the standard assay, which is 0.2 % of acetylacetone-cleavage activity (results not shown). In summary, these data show clearly that Dke1 is an oxygenase with a novel substrate specificity.

DISCUSSION

Dke1, the primary enzyme of the acetylacetone-degradation pathway

We have isolated the initial enzyme of the acetylacetone degradation pathway in *A. johnsonii* (DSMZ ID no. 98-849). This

Scheme 1   Proposed acetylacetone pathway of the isolated *A. johnsonii* strain

Based on the products found with acetylacetone conversion by purified and partially purified nDke1 as well as by cell extract, a potential pathway for acetylacetone degradation in *A. johnsonii* is proposed. In the initial step, acetate and methylglyoxal are formed, catalysed by Dke1. As found with partially purified enzyme, this cleavage reaction is followed by the conversion of methylglyoxal into lactate, a reaction that is typically catalysed by glyoxalases. When using crude cell extracts, lactate is further oxidized to pyruvate by a putative dehydrogenase.

bacterial strain is, to our knowledge, the first reported organism growing with acetylacetone as the sole carbon source. The enzyme Dke1, which has been characterized in this study, initiated the degradation of acetylacetone, cleaving it to acetate and methylglyoxal. When cell extracts from induced *A. johnsonii* cells were employed, the latter metabolite was converted into lactate that was subsequently oxidized to pyruvate. Based on these findings we suggest a pathway that is shown in Scheme 1. Acetylacetone degradation is initiated by its oxygenative cleavage to acetate and methylglyoxal, catalysed by Dke1, and proceeds via the conversion of methylglyoxal into lactate, a reaction that is typically found with glyoxalases. Subsequently, lactate is oxidized to pyruvate by a putative lactate dehydrogenase. The overall reaction is the conversion of acetylacetone into acetate and pyruvate, both being growth substrates for the *A. johnsonii* strain of this study.

Dke1, a novel oxygenase

Dke1 is a novel oxygenase in many respects. Firstly, its apparent physiological role, the detoxification of acetylacetone by its oxygenative cleavage, has not been described before. Secondly, Dke1 has catalytic properties that are so far unique. It is the first reported enzyme that oxygenatively cleaves β-dicarbonyl structures. The consumption of equimolar amounts of O_2 concomitant with the C–C-bond cleavage of the substrate and the formation of two oxygenated products resemble the catalytic activity of C–C-bond-cleaving dioxygenases [24]. Only few exponents of that enzyme type act on aliphatic substrates. Besides carotene and lignostilbene dioxygenases [25,26], which act on non-activated double bonds, a few dioxygenases are known to cleave α-hydroxyketone structures, such as 2,4′-dihydroxyacetophenone dioxygenase from *Alcaligenes* sp. 4HAP [27], aci-reductone dioxygenase [28,29] and quercetin dioxygenase [30]. An oxygenase that is specific for β-dicarbonyl structures is novel.

Finally, the amino acid sequence of Dke1 does not show traceable active-chain similarity with any reported oxidative enzymes and lacks annotated consensus motifs and patterns of described oxygenase families. Considering that histidines are likely candidates to be involved in metal ligation and are actually the predominant metal-binding residues in dioxygenases [24,30,31], we focused on the investigation of the histidine-containing sequence stretches of Dke1. However, by comparison with the reported and potential metal-binding sites of other dioxygenases, no conserved patterns could be identified that might have allowed a prediction as to which of the five histidines of Dke1 is involved in binding. Sequence alignments of structurally characterized dioxygenases with Dke1 based on secondary structure prediction methods were also inconclusive. While this article was undergoing revision, the three-dimensional structure of Dke1 was solved, showing Dke1 to be a member of the cupin superfamily (G. Stranzl and C. Kratky, personal communication). This superfamily, besides a variety of proteins, comprises quercetin dioxygenase and homogentisate 1,2-dioxygenase, of which the structures have recently been solved. However, in spite of overall similarity in folded structure, these dioxygenases are very divergent in terms of function, showing enormous differences with respect to their physiological roles and substrate specificities.

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