Amino Acids Allosterically Regulate the Thiamine Diphosphate-dependent α-Keto Acid Decarboxylase from Mycobacterium tuberculosis\*\( ^{6}\)\(^{6}\)

The gene \(rv0853c\) from \(Mycobacterium tuberculosis\) strain H37Rv codes for a thiamine diphosphate-dependent α-keto acid decarboxylase (\(MtKDC\)), an enzyme involved in the amino acid degradation via the Ehrlich pathway. Steady state kinetic experiments were performed to determine the substrate specificity of \(MtKDC\). The mycobacterial enzyme was found to convert a broad spectrum of branched-chain and aromatic α-keto acids. Stopped-flow kinetics showed that \(MtKDC\) is allosterically activated by α-keto acids. Even more, we demonstrate that also amino acids are potent activators of this thiamine diphosphate-dependent enzyme. Thus, metabolic flow through the Ehrlich pathway can be directly regulated at the decarboxylation step. The influence of amino acids on \(MtKDC\) catalysis was investigated, and implications for other thiamine diphosphate-dependent enzymes are discussed.

Over the past years numerous thiamine diphosphate-dependent (ThDP)\(^5\) α-keto acid decarboxylases have been identified. These enzymes catalyze the non-oxidative decarboxylation of α-keto acids to aldehydes. The elementary steps of the catalytic cycle of these enzymes are identical. Initially, the cofactor is activated by deprotonation of its C2 atom. Subsequently, the generated cofactor ylide attacks the α-carbonyl atom of the substrate, yielding a tetrahedral pre-decarboxylation intermediate. This intermediate is decarboxylated, resulting in the second resonance-stabilized carbanion/enamine intermediate. Finally, the enamine intermediate is protonated, and the reaction product (aldehyde) is released (1).

Based on their substrate specificity, non-oxidative ThDP-dependent α-keto acid decarboxylases yielding aldehydes can be subdivided into various groups. As a rule, substrate specificity reflects the biological function of the enzyme. Pyruvate decarboxylases (PDCs) function in the alcoholic fermentation and convert pyruvate to acetaldehyde (2). Benzoylformate decarboxylases are found in many microorganisms and catalyze the decarboxylation of benzoylformate to benzaldehyde, the third reaction in the mandelate pathway (3, 4). Indolepyruvate decarboxylases (IPDCs) and phenylpyruvate decarboxylases are key enzymes in the biosynthesis of the plant hormones indoleacetic acid and phenylacetic acid, which are derived from the aromatic amino acids tryptophan and phenylalanine, respectively (5, 6). The conversion of a broad spectrum of α-keto acids is characteristic for the branched-chain α-keto acid decarboxylases. In addition to α-keto acids derived from branched-chain amino acids (leucine, isoleucine, and valine), aromatic α-keto acids are used as substrates, too (7, 8). The branched-chain α-keto acid decarboxylases are important enzymes in the Ehrlich pathway (9, 10). The first step of the Ehrlich pathway is the transamination of amino acids to their corresponding α-keto acids. Subsequently, the α-keto acids are decarboxylated to aldehydes. This quasi-irreversible reaction is catalyzed by a ThDP-dependent α-keto acid decarboxylase, which converts a broad spectrum of substrates (8, 9). Depending on the redox state of the cell, aldehydes are either oxidized to fusel acids or reduced to fusel alcohols (Fig. 1). The Ehrlich pathway, also denoted as amino acid fermentation, is best investigated in yeast (9–11). Vuralhan et al. (9) showed that \(Saccharomyces cerevisiae\) transcriptionally up-regulates \(aro10\), the gene encoding the corresponding α-keto acid decarboxylase in yeast, when grown on amino acids phenylalanine, leucine, or methionine as nitrogen source, whereas the mRNA amount of other ThDP-dependent decarboxylases (PDC1, PDC5, PDC6, THI3) was not affected. However, a poor correlation between the transcription level and enzyme activity in cell free extracts indicated that the ARO10 activity is not solely regulated by the amount of enzyme, and an additional posttranscriptional regulation of the decarboxylase activity was proposed (9, 11).

Transcriptional and posttranscriptional regulation mechanisms are also described for some pyruvate decarboxylases, which are key enzymes in the anaerobic fermentation of glucose, yielding ethanol and carbon dioxide. It was shown that expression of PDC from \(Kluyveromyces lactis\) (KIPDC) is
induced by glucose and low oxygen concentrations at the transcriptional level (12, 13). Additionally, an allosteric activation by the substrate pyruvate was described for \( \text{KIPDC} \) (14), as it is known for many other PDCs (15–20). Whereas transcriptional regulation requires time and a high energy input, allosteric regulation is a short-term and quick response to fluctuating conditions in the cell. The molecular basis of substrate activation has long been studied for PDCs from yeast. One of the basic kinetic models of substrate activation of PDCs consists of two steps and describes the rapid binding of an activator molecule to the regulatory site, which triggers the slow isomerization of an inactive enzyme to a catalytically active enzyme (15, 21).

Another kinetic model, based on extensive studies conducted to establish a signal transfer pathway from the substrate bound at the regulatory site to the cofactor ThDP at the active site (27–31). Another kinetic model, based on extensive studies on the pH dependence of activation, favors the existence of various activated enzyme states (32, 33). For an excellent review on PDC activation, see Schowen (34).

In this study we provide kinetic evidence that a ThDP-dependent \( \alpha \)-keto acid decarboxylase, which participates in amino acid degradation via the Ehrlich pathway, is subject to allosteric activation. We demonstrate that \( \alpha \)-keto acids and, most importantly, also the corresponding amino acids are allosteric activators of enzyme activity. Binding of amino acids at the regulatory site and binding of substrates at the active site show positive cooperativity.

To determine which elementary steps in MtKDC catalysis are affected by amino acids, the cofactor activation and distribution of reaction intermediates was analyzed. Eventually, implications for other non-oxidative ThDP-dependent \( \alpha \)-keto acid decarboxylases are discussed.

**EXPERIMENTAL PROCEDURES**

**Reagents**—All substrates (except \( \alpha \)-ketovalerate and \( \alpha \)-ketocaproat), ThDP, \( \pi \)-amino acids, and alcohol dehydrogenase from yeast and horse liver were obtained from Sigma Aldrich and Fluka (Seelze, Germany). \( \pi \)-Amino acids and NADH were obtained from AppliChem (Darmstadt). Synthesis of \( \alpha \)-ketovalerate and \( \alpha \)-ketocaproat were performed according to Fischer (35).

**Cloning**—Genomic DNA from *M. tuberculosis* strain H37Rv was used as the PCR template. The following gene-specific primers containing 5'-Ndel and 3'-HindIII restriction sites (underlined in the primer sequence) were designed. An additional GCT codon was introduced in the forward primer 5'-AAAACATATGGCTGTGACACCCCCAGAAGACGATGACCTGCAG-3' to increase the expression efficiency. The reverse primer 5'-AAAAAAGCTTTATCATCTGCGGCGCATGGATCCACGAGTTGG-3' contains one extra stop codon to suppress the production of the C-terminal His\(_6\) tag included in the cloning vector. The amplified rv0853c gene was cloned into the PCR-BluntII TOPO vector and transformed into One Shot TOP10 cells (Invitrogen). Plasmid DNA was isolated, and the identity of the cloned fragment was confirmed by sequencing. MtKDC coding plasmid DNA was finally digested and ligated into the Ndel/HindIII restriction sites of expression vector pET22b(+) (Novagen, Darmstadt).

**Protein Expression and Inclusion Body Isolation**—For gene expression, *Escherichia coli* Rosetta 2 (DE3) (Novagen) transformants were grown at 30 °C in 2\( \times \)YT medium containing 50 \( \mu \)g/ml ampicillin. Gene expression was induced at an \( A_{600} \) of 0.8–1.0 by the addition of 0.5 mM isopropyl-\( \beta \)-d-thiogalactopyranoside. Cells were harvested after 8 h, flash-frozen, and stored at \(-80^\circ\)C. Although various growth conditions have been tested (varying temperature and isopropyl-\( \beta \)-d-thiogalactopyranoside concentration using various *E. coli* expression strains), formation of inclusion bodies could not be prevented. Inclusion bodies were isolated as described by Rudolph et al. (36).

**Inclusion Body Solubilization, Refolding, and Purification**—Inclusion bodies were suspended in 8 M urea, 100 mM Tris, pH 8.0, 100 mM dithioerythritol, and 1 mM EDTA and stirred for 2 h at room temperature. The remaining insoluble material was removed by centrifugation (25 min, 70,000 \( \times \) g). Denatured
Amino Acids as Allosteric Regulators of Enzyme Activity

protein was dialyzed twice against 6 M urea, 100 mM Tris/HCl pH 8.0, and 1 mM EDTA. Insoluble material was again separated by centrifugation. The supernatant was diluted to 2 mg/ml with 6 M urea, 100 mM Tris/HCl, pH 8.0, and 1 mM EDTA and refolded while dialyzing against 50 mM Tris/HCl pH 7.5, 1 mM EDTA, and 1 mM dithioerythritol. After refolding, 200 mM ammonium sulfate was added, and the solution was incubated at 30 °C for 20 min. The precipitated protein was separated by centrifugation, and the supernatant was dialyzed against 20 mM Tris/HCl pH 7.5, 1 mM EDTA, and 1 mM dithioerythritol. The refolded protein was then applied to an ion exchange column (Fractogel TMAE, column 26 × 200 mm). Elution was performed using a linear gradient of 180 ml of 0–50% 500 mM NaCl in 20 mM Tris, pH 7.5. The MtKDC-containing fractions, eluting at 100–200 mM NaCl, were pooled and concentrated. MtKDC was further purified to >95% homogeneity by gel filtration on Superdex 200 (GE Healthcare, column 16 × 600 mm), equilibrated with 50 mM Mes/NaOH, pH 6.5, and 150 mM ammonium sulfate. The identity of the protein was confirmed by combination of tryptic digestion and matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

Characterization of Substrate Derivatives—Keto-enol tautomeration of α-ketoisocaprate and α-ketoisovalerate, dissolved in 100 mM acetate, pH 6.0, and 10% (v/v) deuterium oxide, was investigated by 1H NMR (H, H COSY) and 13C NMR (13C,1H HSQC (heteronuclear single quantum correlation), Attached Proton Test). The amount of keto form, enol form, and hydrate form was deduced from the peak areas of the 1H NMR spectra. All experiments were performed on a Bruker Avance ARX 400 NMR spectrometer (supplemental Figs. 1 and 2).

Protein Concentration—Samples containing ThDP were determined with Bradford assay using bovine serum albumin as standard (37). Otherwise, the protein concentration was calculated from UV spectra using the calculated molar extinction coefficient of 55,350 M–1cm–1 at 280 nm for the MtKDC monomer (see ExPASy).

Kinetic Measurements—Measurements including 1H NMR experiments were performed at 30 °C in 100 mM Mes/NaOH, pH 6.5. The decarboxylation of pyruvate, α-ketobutyrate, α-ketovalerate, and α-ketocaproate was monitored using a coupled enzymatic assay with alcohol dehydrogenase from yeast as the auxiliary enzyme (4, 39). For the aromatic α-keto acids indolepyruvate, phenylpyruvate, and 4-hydroxyphenylpyruvate, alcohol dehydrogenase from horse liver was used as auxiliary enzyme (40). The conversion of NADH was followed at 340 and 366 nm. The decarboxylation of the branched-chain α-keto acids (α-ketovalerate, α-keto-β-methylvalerate, and α-ketoisocaprate) was measured by using a direct assay, monitoring the n→π* transition of the substrate carbonyl group. For the calculation of specific activities, the following molar extinction coefficients were determined under standard measurement conditions: α-ketoisocaprate 32.5 M–1cm–1 at 314 nm, α-keto-β-methylvalerate 33.0 M–1cm–1 at 316 nm, α-ketoisocaproate 25.4 M–1cm–1 at 317 nm. Catalytic constants of indolepyruvate conversion were calculated, taking the slow keto-enol equilibrium into account. The effective fraction of the reactive keto form under measurement conditions was 85% for indolepyruvate (40). For 4-hydroxyphenylpyruvate and phenylpyruvate, 87.5 and 98% keto form are found in equilibrium. However, because the equilibria establish very rapidly for these two substrates, the nominal molar concentrations have been used for data analysis. For branched-chain α-keto acids, most of the substrate exists in the keto form (α-ketoisovalerate, 95–96% keto form and 4–5% hydrated form; α-ketoisocaproate, 98% keto form and 2% hydrated form; see supplemental Figs. 1 and 2). One unit of catalytic activity is defined as the amount of enzyme converting 1 μmol of α-keto acid/min at 30 °C. kcat values were determined per monomer using a molecular mass of 59,783 Da (calculated from the deduced amino acid sequence).

Stopped-Flow Measurements—Substrate activation and amino acid activation were analyzed using a stopped-flow spectrophotometer (SX18MV, Applied Photophysics, Surrey) and the standard buffers described above. A solution containing substrate (for substrate activation analysis) or substrate and amino acid (for amino acid activation studies) was mixed in a 1:1 ratio with a solution containing the auxiliary enzyme alcohol dehydrogenase (0.5–0.9 units/ml), NADH (0.7–1.9 mM), and MtKDC (0.27–4.9 μM). Progress curves were fitted according to Equation 1 (A is absorbance at time t, and A0 is initial absorbance), thereby obtaining the observed first order rate constant for the substrate activation process (kobs, the initial velocity (v0), and the steady state velocity (vSS)).

\[
A = A_0 - v_{SS} \cdot t + \frac{v_{SS} - v_0}{k_{obs}} \cdot (1 - e^{-k_{obs} \cdot t}) \quad (\text{Eq. 1})
\]

1H NMR Experiments—To investigate the influence of activators on the deprotonation rate constant at the C2 atom of enzyme-bound ThDP, H/D exchange kinetics were monitored in presence and absence of an amino acid at 10 mg of MtKDC/ml, 30 °C, and pH 6.5 (41). Additionally, distributions of enzyme-bound covalent ThDP intermediates were determined in the absence and presence of an amino acid according to the method described in Tittmann et al. (42) at 15 mg of enzyme/ml. In both experiments L-leucine was chosen as the amino acid, because it acts as a strong activator in conventional kinetic experiments. For each reaction run, the enzyme was preincubated with 5 mM L-leucine. For reaction intermediate analyses, measurements were started by the addition of 60 mM pyruvate and stopped by acid quench after 5 s. In the absence of L-leucine, the reaction does not reach the (final) steady state within this reaction time. Because the enzyme converts the substrate very rapidly at the required high protein concentration, the reaction time cannot be significantly expanded beyond 5 s. This restriction prevented the calculation of microscopic rate constants for the catalytic cycle. However, semiquantitative analyses have been carried out.

The Kinetic Model—A two-site model has been developed to fit the kinetic data (Fig. 2). Formally it is composed of two sections. In the absence of the effector X (amino acids in our study) only the species of the upper branch (E,S, E,S, SEα, SEα, SEα, SEα, S) are populated. Substrate molecules may either bind to the active site (formation of E,S) or to the regulatory site (formation of SEα) of the initial enzyme state. Binding at the regulatory site triggers a conformational switch to more active enzyme forms (SEα, SEα, S). This isomerization step is rate-limiting for the acti-
Amino Acids as Allosteric Regulators of Enzyme Activity

Obviously it holds \( \frac{k^{\prime}_{\text{cat}}}{k^{0}_{\text{M}}} = k^{0}_{\text{cat}}/k_{1} \). The complex dependence of \( k_{\text{obs}} \) on \( S \) after Equation 3 can run through minima or maxima but may also account for quasi-hyperbolic \( k_{\text{obs}}/S \)-plots (14).

In the presence of the effector \( X \) the entire scheme of Fig. 2 theoretically becomes relevant. In this case, \( S \) and \( X \) compete for the regulatory site, whereas \( X \) is not supposed to bind to the active site. Because \( X \) actuates conformation changes (steps \( k_{4} \) and \( k_{6} \)) leading to the activated species \( XE_{a} \) and \( XE_{a}S \), it acts as a heterotropic activator. Because of the two independent activation pathways (substrate driven and amino acid driven activation), progress curves should display two lag phases. Empirically, this is not the case. This leads to the assumption that in the presence of amino acids and moderate substrate concentrations, the amino acid-driven pathway dominates, and hence, the sequence \( SE_{i} \rightleftharpoons SE_{o} \rightleftharpoons SE_{a}S \) might be neglected.

The intrinsic mathematical properties of the model are outlined in the section below. The following assumptions are made. (i) The substrate binding steps are fast compared with the isomerization steps \( SE_{i}/SE_{o}, XE_{i}/XE_{a}, \) and \( XE_{a}S/\)XE_{a}. (ii) In the presence of amino acid the transition of \( SE_{i} \) to \( SE_{a} \) is suppressed. All relevant differential equations can be added to give

\[
\begin{align*}
\frac{dSE_{i}}{dt} + \frac{dE_{i}}{dt} + \frac{dES}{dt} + \frac{dXE_{i}}{dt} + \frac{dXE_{a}S}{dt} &= -k_{4} \cdot XE_{i} - k_{6} \cdot XE_{i} \\
&+ k_{4} \cdot XE_{a} + k_{6} \cdot XE_{a}S \\
&= (k_{4} \cdot XE_{i} + k_{6} \cdot XE_{i}) - (k_{4} \cdot XE_{a} + k_{6} \cdot XE_{a}S) \\
&= \text{Eq. 6}
\end{align*}
\]

The mass balance equation of all enzyme species reads as

\[
E_{0} = E_{i} \cdot \left(1 + \frac{S}{K_{a}} + \frac{X \cdot S}{K_{a} \cdot K_{3}} + \frac{X}{K_{3}}\right) + E_{a} \cdot \left(1 + \frac{S}{K_{3}}\right) \\
\text{Eq. 7}
\]

Insertion of Equation 7 into Equation 6 yields

\[
\frac{dE_{i}}{dt} = -k_{\text{obs}} \cdot E_{i} + \frac{\gamma \cdot E_{0}}{\alpha \cdot \eta} \\
\text{Eq. 8}
\]

with

\[
k_{\text{obs}} = \frac{\left(\frac{k_{4} + k_{6} \cdot S}{K_{3}}\right) \cdot X}{\left(1 + \frac{S}{K_{a}} + \frac{X \cdot S}{K_{a} \cdot K_{3}} + \frac{X}{K_{3}}\right)} + \frac{\left(k_{4} \cdot XE_{a} + k_{6} \cdot XE_{a}S\right)}{K_{3} + S} \\
\text{Eq. 9}
\]

\[
\nu = k_{4} \cdot k_{a} + k_{6} \cdot S/K_{3}, \quad \eta = 1 + S/K_{3}, \quad \alpha = 1 + S/K_{a} + S/K_{1} + X/K_{a} + X \cdot S/K_{2} \cdot K_{a}.
\]

As a solution to Equation 8 one obtains

\[
E_{i} = (E_{0} - E_{eq}) \cdot e^{-k_{\text{cat}} \cdot \tau} + E_{eq} \\
\text{Eq. 10}
\]

with \( E_{\text{eq}} = E_{eq}/\alpha \) and \( E_{eq} = \gamma \cdot E_{eq}/\alpha \cdot \eta \cdot k_{\text{obs}} \cdot E_{\text{eq}} \). \( E_{\text{eq}} \) is the concentration of the enzyme species \( E_{i} \) immediately after the rapid equilibration between the species \( E_{i}, E_{s}, XE_{a}, \) and \( XE_{a}S \), which occurs after mixing the enzyme with \( S \) and \( X \). Because the isomerization steps \( (k_{4}, k_{6}, k_{a}, k_{b}) \) are considered to be slow, virtually no \( XE_{a} \) or \( XE_{a}S \) has been formed at this stage. The index “eq” relates to the final equilibrium of all enzyme species. Equation 10 can be rearranged to give
Amino Acids as Allosteric Regulators of Enzyme Activity

\[ X_{E,S} = X_{E,S} \text{eq} \cdot (1 - e^{-k_{24} S}) \]  
(Eq. 11)

which in turn can easily be converted to

\[ v = v_0 + \left( v_{SS} - v_0 \right) \cdot (1 - e^{-k_{24} S}) \]  
(Eq. 12)

with

\[ v_{SS} = \frac{V_{max}}{K_m} \cdot \frac{S}{K_m + S} \]  
(Eq. 13)

\[ V_{max}^{app} = \frac{k_{cat} \cdot E}{1 + \frac{K_f}{K_m} + \frac{1}{K_m} \cdot \frac{1}{K_f \cdot K_m}} \]  
(Eq. 14)

and

\[ K_m^{app} = \frac{K_1 \left[ 1 + \frac{X}{K_m} \left[ \frac{1}{K_m} + \frac{1}{K_a} \right] \right]}{1 + \frac{K_1}{K_A} + \frac{X}{K_m} \cdot \left[ \frac{1}{K_m} + \frac{1}{K_a} \cdot \frac{1}{K_m \cdot K_a} \right]} \]  
(Eq. 15)

Notably, the analytical expressions for \( v_0 \) and \( v_{SS} \) in Equation 12 differ from those in Equation 2. Most importantly, the amino acid driven mechanism predicts Michaelis-Menten behavior as only one substrate molecule is bound at the working enzyme. Integration of Equation 12 reproduces Equation 1. Equation 9 defines a surface function, which is spanned over the XS plane. As seen from the slightly rearranged formulation in Equation 16, \( k_{obs} \) depends always hyperbolically on \( X \).

\[ k_{obs} = k^b + k_{max} \cdot X \]  
(Eq. 16)

with

\[ k^b = \frac{k_{-4} \cdot K_S + k_{-6} \cdot S}{K_1 + S} \]  
(Eq. 17)

\[ k_{max} = \frac{k_4 \cdot K_S + k_6 \cdot S}{K_2 + S} \]  
(Eq. 18)

and

\[ K_{obs} = K_a \cdot K_2 \cdot \frac{1 + S/K_2}{K_2 + S} \]  
(Eq. 19)

\( k_{max} \) is the rate constant for the activation in forward direction at a given substrate concentration under amino acid saturation. \( k^b \) relates to the reverse reaction. \( K_{obs} \) represents an apparent dissociation constant of the effector \( X \) for the initial enzyme state, again relevant for a defined substrate concentration. Technically, \( K_{obs} \) is the half-saturation constant of the \( k_{obs} \) dependence at a given substrate concentration. The dependence of \( k_{obs} \) on \( S \) is potentially more complex. Depending on the individual values of the microscopic constants, \( k_{obs} \) may display upward or downward curvatures over the substrate domain. In theory, Equations 16–19 offer a straightforward method for data evaluation. A comprehensive kinetic analysis would require the determination of \( k^b, k_{max} \) and \( K_{obs} \) for several sufficiently varying substrate concentrations. Subsequently, the three apparent constants \( k^b, k_{max} \) and \( K_{obs} \) should be replotted as functions of \( S \). Ideally, \( k^b \), \( k_{max} \) and \( K_{obs} \) could be extracted from a plot of \( k_{max} \) versus \( S \), whereas analysis of \( K_{obs} \) would allow the estimation of \( K_1 \), \( K_2 \), and \( K_3 \). Plotting of \( k_{max} \) versus \( S \) would finally yield \( k_{-4}, k_{-6}, \) and \( K_4 \). In the current study this approach remained restricted to the \( S/X \) couple benzyloformate/L-leucine. In all other cases we merely report the net rate constants \( k^b, k_{max} \) and \( K_{obs} \) at a constant substrate concentration of 5 mm instead.

For the comparison of the activation potential of \( \alpha \)-keto acids and amino acids, it is expedient to introduce specificity constants for activation, defined in analogy to catalytic efficiencies \( k_{cat}/S_{0.5} \). At low concentrations the activation potential of amino acids can be characterized by the second-order rate constant \( k_{max}/K_{obs} \). The activation potential of substrates is given by the ratio of \( k_{iso}/K_{cat} \), which is numerically equal to \( k_{iso}/K_A \) (see Equation 3).

RESULTS AND DISCUSSION

Cloning, Expression, and Purification—The mycobacterial gene rv0853c encoding a putative PDC or IPDC was amplified from genomic DNA. The \( MtKDC \) was expressed as inclusion bodies, refolded, and purified to homogeneity using anion exchange chromatography and gel filtration. Approximately 50 mg of protein were obtained from 6 liters of cell culture.

Substrate Specificity—As based on amino acid sequence comparison, \( MtKDC \) was originally proposed to be a PDC or IPDC (UCLA-DOE Institute for Genomics and Proteomics). To classify purified \( MtKDC \) on empirical grounds, we determined the catalytic constants for the conversion of 11 aliphatic, aromatic, and branched-chain \( \alpha \)-keto acids. Table 1 summarizes the calculated values for \( S_{0.5} \) and \( k_{cat} \). Pyruvate was converted with the lowest catalytic efficiency \( (k_{cat}/S_{0.5}) \) and displayed a weak substrate inhibition (Fig. 3C). The highest catalytic efficiencies were found for indolepyruvate and \( \alpha \)-ketoisocaproate. In general, the order of the \( S_{0.5} \) values point to preferred binding of hydrophobic substrates, with the possible exception of benzyloformate. The highest \( k_{cat} \) values were indeed observed for branched-chain \( \alpha \)-keto acids. Thus, \( MtKDC \) appears to be optimized for the conversion of aromatic and branched-chain \( \alpha \)-keto acids, which contrasts strikingly to PDCs that prefer pyruvate as substrate (44). A similar substrate spectrum was found for other ThDP-dependent enzymes partaking in amino acid degradation, e.g., for the branched-chain \( \alpha \)-keto acid decarboxylase from \( Lactococcus lactis \) (KdcA) (7, 8) and for the \( \alpha \)-keto acid decarboxylase ARO10 from \( S. cerevisiae \) (9). KdcA has the highest catalytic specificities for phenylpyruvate and \( \alpha \)-ketoisovalerate but the lowest for pyruvate (7). ARO10 was shown to convert phenylpyruvate, \( \alpha \)-ketoisovalerate, \( \alpha \)-keto-\( \beta \)-methylvalerate, and \( \alpha \)-ketoisocaproate (9). Additionally, we show also that EcIPDC decarboxylates phenylpyruvate as well as branched-chain and extended aliphatic \( \alpha \)-keto acids (supplemental Table 1). Taken together, ThDP-dependent enzymes involved in amino acid degradation via the Ehrlich pathway display a broad substrate spectrum, some preference for hydrophobic substrates, and a low specificity for pyruvate.
### TABLE 1

Catalytic constants for the decarboxylation of different α-keto acids by MtKDC

| Substrate                  | $S_{0.5}$ $k_{cat}$ | $k_{cat}/S_{0.5}$ | $n$ | $k_{cat}^{obs}$ | $K_0^{obs}$ |
|----------------------------|---------------------|-------------------|-----|-----------------|-------------|
| Indole pyruvate            | 0.26 ± 0.12         | 2.98 ± 0.11       |      | 11.46           | 1.22        |
| α-Ketoisocaproate          | 2.90                |                   |      | 10.86           | 1.30        |
| Phenyl pyruvate            | 0.83 ± 0.08         | 7.50 ± 0.10       | 9.03 | 1.41            | 1.47        |
| α-Keto caproate            | 0.92 ± 0.22         | 7.06 ± 0.01       | 7.67 | 1.61            | 1.25        |
| α-Keto valerate            | 1.31 ± 0.24         | 6.95 ± 0.04       | 5.30 | 1.33            | 1.13        |
| 4-Hydroxyphenylpyruvate    | 0.93 ± 0.18         | 3.89 ± 0.04       | 4.18 | 1.52            | 1.21        |
| α-Keto-β-methyl valerate   | 6.68 ± 0.97         | 19.75 ± 1.21      | 2.96 | 1.32            | 10.05       |
| Benzyloformate             | 8.25 ± 0.78         | 18.57 ± 0.47      | 2.25 | 1.96            | 7.05        |
| α-Keto isovalerate         | 24.47 ± 3.06        | 28.35 ± 2.19      | 1.16 | 1.21            | 26.11       |
| α-Keto butyrate            | 14.17 ± 3.95        | 5.98 ± 0.33       | 0.42 | 1.04            | 3.68        |
| Pyruvate*                  | 98.89 ± 37.48       | 2.14 ± 0.21       | 0.02 | 1.02            | 0.40        |

*Weak substrate inhibition ($K_i = 124$ ms) was detected only for pyruvate.

#### FIGURE 3. Kinetics of MtKDC activation in the absence of amino acids.

A) Original (open circles and triangles) and differentiated (closed circles) progress curves of catalysis at different pyruvate concentrations (1, 10 mM; 2, 15 mM; 3, 25 mM; 4, 40 mM). B) Semilogarithmic plots of the dependence of the activation rate constant ($k_{cat}$) on substrate concentration for different α-keto acids (circles, pyruvate; squares, α-ketoisocaproate; diamonds, phenylpyruvate; triangles, phenylisocaproate). C) Semilogarithmic plots of the dependence of steady state activity ($V_{max}$) on substrate concentration for different α-keto acids (circles, pyruvate; squares, α-ketoisocaproate; diamonds, phenylpyruvate; triangles, phenylisocaproate). D) Semilogarithmic plots of the dependence of initial activity ($v_0$) on substrate concentration for different α-keto acids (circles, pyruvate; squares, α-ketoisocaproate; diamonds, phenylpyruvate; triangles, phenylisocaproate).

Interestingly, MtKDC exhibits a sigmoid dependence of its catalytic activity on substrate concentration. Whereas the sigmoidicity is distinctly pronounced for benzoylformate, α-keto-caproate, 4-hydroxyphenylpyruvate, and phenylpyruvate, it is much weaker in case of the other α-keto acids analyzed (see Table 1 and supplemental Fig. 3). Sigmoid dependence of the catalytic activity on the substrate concentration generally indicates substrate activation behavior.

**Substrate Activation Behavior**—Progress curves displayed distinct lag phases, pointing to an allosteric substrate activation mechanism (Fig. 3A). Because the observed $k_{cat}$ values are at least one order of magnitude higher than the $k_{obs}$ values associated with the lag phases, catalytic steps cannot be rate-limiting for these transients. Such behavior was described for all PDCs investigated so far (14–16), except for the enzyme from *Zymomonas mobilis* (45). A basic kinetic model for this phenomenon was originally developed by Hübner et al. (15) and extended later by Alvarez et al. (21). An alternative model with various activated enzyme states was advanced by Sergienko and Jordan (32, 33).

Stopped flow transients possessed significant initial velocities ($v_0$) (Fig. 3, A and B). A similar phenomenon was found for PDC from *Pisum sativum* (16). In contrast, initial velocities close to zero are observed for other substrate-activated PDCs such as ScPDC and KtPDC. These enzymes are, thus, potentially inactive in the absence of substrate (14, 15, 46). The observed initial velocities of MtKDC and their hyperbolic dependence on substrate concentration (Fig. 3C, Table 1, see also supplemental Fig. 3) cannot be explained by the kinetic model of Alvarez et al. (21). Therefore, we postulate an initial enzyme state that is partially active in the absence of the substrate (Fig. 2). The addition of substrates triggers the slow transition of the enzyme with a low basic activity ($SE_{E_0}$) into a fully activated enzyme species ($SE_{E_1}$, $SE_{E_2}$, $SE_{E_3}$). Alternatively, initial activities could be described by an equilibrium between inactive and active enzyme species, which undergoes a shift toward the activated state upon the addition of substrate. So far, this is not substantiated by our data but cannot be completely ruled out at the moment. Table 1 includes a sur-
ve of empirical $k_{cat}$ values of the initial enzyme state ($k_{cat}^0$) for several substrates as well as their respective $K_{V}^0$ values. It should be noted that the given $k_{cat}^0$ are drawn from fittings of $v_0$ versus $S$ according to Equation 4, therefore not being identical to the microscopic $k_{cat}^0$ values, which will be somewhat higher. The strongest activation effects in terms of $k_{cat}^0/k_{cat}^0$ have been observed for indolepyruvate (7.45), $\alpha$-ketocarate (6.15), and $\alpha$-ketocaprate (5.65).

Because of limited solubility of the substrates, it was impossible to reach the saturation ranges of the $k_{obs}/S$ plots for any of the substrates, apart from pyruvate (Fig. 3D). However, a marked curvature indicating substrate saturation behavior is seen for most substrates (see supplemental Fig. 4). Thus, the associated fitting errors of the maximum reaction rate constant ($k_{iso}$) and the half-saturation concentration of the activation rate ($K_a$) are relatively high. In some cases these parameters could not have been determined at all (see Table 2 and supplemental Fig. 4). Nevertheless, in each case the ratio of $k_{cat}^0/k_{cat}^0$ could be calculated with sufficient accuracy from the initial slope of the concentration dependence of $k_{obs}$ (Table 2). These values represent a measure of the activation potentials of substrates. Table 2 demonstrates that aromatic $\alpha$-keto acids exert a considerable activating effect on MtKDC, with indolepyruvate being the most efficient activator. In contrast, pyruvate is the weakest $\alpha$-keto acid activator for MtKDC. Under equivalent conditions, the activation potential ($k_{cat}^0/K_a = k_{iso}/K_a$) of pyruvate is significantly higher for substrate-activated PDCs ($k_{cat}^0$, 0.215 mm$^{-1}$s$^{-1}$; ScPDC, 0.070 mm$^{-1}$s$^{-1}$; PsPDC, 0.375 mm$^{-1}$s$^{-1}$) (14). As evidenced in a series of publications, Cy321 of ScPDC is the starting point for the information transfer in the process of substrate activation (47–49). Interestingly, this amino acid is not conserved at the equivalent position of MtKDC (supplemental Fig. 5). As a consequence, the molecular activation mechanism of MtKDC should differ from that of ScPDC.

Amino Acid Activation—It is well known that many bacteria catabolize aromatic and branched-chain amino acids via the Ehrlich pathway and that the rate-limiting step of this route is catalyzed by a ThDP-dependent decarboxylase (8, 50). Thus, we investigated whether precursors (amino acids) and end products (fuel alcohols and fuel acids) of the Ehrlich pathway affect MtKDC activity. Whereas tryptophol (fuel alcohol), phenylacetic acid, and indoleacetic acid (fuel acids) had no effect, we could clearly demonstrate that amino acids are potent activators of MtKDC (Fig. 4A). We tested the effect of L-leucine, L-valine, D-valine, L-isoleucine, L-phenylalanine, D-phenylalanine, L-tyrosine, L-tryptophan, and L-alanine. All these amino acids except L-tryptophan produce a distinct shortening of the lag phases of the progress curves. A hyperbolic dependence of the activation rate constant $k_{obs}$ on the amino acid concentration was generally found (Fig. 4, B and C), except for L-tyrosine and D-phenylalanine due to their insufficient solubility. These findings relate to the amino acid section of the kinetic model of MtKDC in Fig. 2, postulating that the binding of amino acid at the regulatory site triggers a slow isomerization of enzyme species with low activity ($X_E$, $X_E$) to completely activated enzyme species ($X_{cat}$, $X_{cat}$). The kinetic constants for amino acid activation were calculated according to Equation 16 and are summarized in Table 3. A comparison of the activation potential of amino acids in terms of $K_{cat}/K_{V}^0$ revealed that L-leucine and L-isoleucine are the strongest activators for MtKDC. A significant lower affinity of MtKDC was shown for L-valine as compared with L-valine (Fig. 4B, Table 3), indicating that the configuration at the Ca-atom is important for the binding of ligands to the regulatory site, too.

As anticipated for allosteric activators, the presence of amino acids at saturation concentration shifts the sigmoid character of the $v/S$-plots toward Michaelis-Menten behavior. Fig. 4D illustrates the dependence of the reaction rate on the $\alpha$-ketoisovalerate concentration in the absence and the presence of two different L-valine concentrations. The transition from a sigmoid curve to a hyperbolic one is accompanied by decreasing $S_{0.5}$ values and increasing $k_{cat}$ values. In absence of L-valine, $k_{cat}/S_{0.5}$ is 1.16 mm$^{-1}$s$^{-1}$, and in the presence of L-valine (50 $\mu$M and 1 mm), $k_{cat}/S_{0.5}$ values are 2.02 and 8.3 mm$^{-1}$s$^{-1}$, respectively. Consequently, amino acids substantially increase the catalytic efficiency of MtKDC for its substrates. Because the empirical $k_{cat}$ values of amino acid activated MtKDC ($k_{cat}$) are only moderately elevated compared with that of the substrate-activated enzyme ($k_{cat}^0$), the major part of this increase of catalytic efficiency rests on the lowered $S_{0.5}$ values.

Interdependence of Substrate and Amino Acid Binding.—To obtain further information on substrate activation and amino acid activation as well as on their interplay, we investigated the dependence of the activation rate on the amino acid concentration at different substrate concentrations. An increase of the substrate concentration resulted in an increase of the amino acid values present the fitting errors.

**TABLE 2**

Microscopic constants for the activation of MtKDC by $\alpha$-keto acids

Values for $k_{cat}^0$, $k_{iso}$, and $K_a$ were determined by fitting the experimental data to Equation 3 (see also supplemental Fig. 4), whereas the values present the fitting errors. $k_{cat}^0/k_{cat}^0$ is a measure of the activation efficiency of the $\alpha$-keto acid and is numerically equal to $k_{iso}/K_a$. For phenylpyruvate, ketocaprate, and benzoylformate this ratio was calculated from the initial slope of the concentration dependence of $k_{cat}^0$.
Amino Acids as Allosteric Regulators of Enzyme Activity

activation of MtKDC by L-leucine as activator and benzoylformate as substrate can be estimated: $K_1 = 126.3 \text{ mM}$, $K_2 = 14.5 \text{ mM}$ (Equation 19, Fig. 5B), $K_{cat} = 0.103 \text{ mM}$ (Fig. 5B), $K_e = 0.89 \text{ mM}$ (Equation 18, Fig. 5C), $k_3 = 0.40 \text{ s}^{-1}$ (Equation 18, Fig. 5C), and $k_5 = 0.11 \text{ s}^{-1}$ (Equation 18).

$^1$H NMR Experiments—To elucidate which reaction steps of the catalytic cycle are specifically affected by allosteric activation of amino acids, we analyzed the H/D exchange at the C2 atom of ThDP as well as the distribution of reaction intermediates formed during the catalysis in absence and presence of amino acids. H/D-exchange experiments clearly show that the C2 deprotonation of the cofactor ThDP is not rate-limiting for catalysis. Moreover, the presence of L-leucine did not substantially increase the rate constant for the H/D exchange ($k_{obs} = 122 \pm 13.7 \text{ s}^{-1}$ without L-leucine and $157 \pm 73.9 \text{ s}^{-1}$ with L-leucine, as compared with the highest $k_{cat}$ value of 31.5 s$^{-1}$, see Table 1). This points again to a fundamental difference between the activation mechanism of PDCs and MtKDC. In contrast, in ScPDC C2 deprotonation of the cofactor is considerably accelerated in the presence of the artificial activator pyruvamide (41).

Finally, we investigated the influence of the strongly activating amino acid L-leucine on the distribution of intermediates, formed in the presence of the poorly activating substrate pyruvate. Our experiments revealed that in the absence of the amino acid, 32% of the enzyme-bound was found as the reaction intermediate 2-hydroxyethyl-ThDP, leaving most of the

acid affinity to MtKDC (Fig. 5, A and B). This positive cooperativity between the binding of the substrate and the amino acid indicates the presence of a regulatory site spatially separated from the active site. In accordance with the measured kinetic data, we propose the existence of a common regulatory site for the keto acids and the amino acids. Furthermore, an increase of the substrate concentration resulted in a decrease of the maximum observed activation rate constant under amino acid saturation (Fig. 5, C and D). Fitting our empirical $k_{obs}$ values according to Equation 16–19, the following constants for the enzyme-bound ThDP unreacted. In the presence of L-leucine, however, 76% of the enzyme-bound cofactor existed in the form of reaction intermediates 2-hydroxyethyl-ThDP (67.5%) and 2-lactyl-ThDP (8.5%) (Fig. 6A). Hence, the catalytic step affected by amino acids precedes or is identical with the formation of the first covalently bound reaction intermediate 2-lactyl-ThDP. Because the formation of the ylide is not affected by the amino acid, the carbonyl addition of the substrate seems to be the target of amino acid activation (Fig. 6B). Furthermore, in the presence of L-leucine the concentration ratio of 2-lactyl-

FIGURE 4. Kinetics of MtKDC activation in the presence of amino acids. A, progress curves of benzoylformate catalysis (5 mM) at different concentrations of L-leucine (1, 0.0 mM; 2, 0.01 mM; 3, 0.05 mM; 4, 0.2 mM; 5, 2 mM). B and C, dependence of the activation rate constant ($k_{obs}$) on amino acid concentration. Experimental data were fitted according to Equation 16. All measurements were performed at 5 mM benzoylformate as substrate. B: closed hexagons, L-leucine; open squares, L-valine; cross-hairs, D-valine. C: closed diamonds, L-tryptophan; closed circles, L-alanine; filled triangles, L-phenylalanine; filled inverted triangles, D-phenylalanine; closed hexagons, L-leucine. For better visualization and comparison of various amino acids, a semilogarithmic scale was used. D, influence of L-valine on the steady state rates of α-ketoisovalerate decarboxylation. MtKDC was preincubated with 0.05 mM (open circles) and 1 mM (closed diamonds), respectively; closed circles, without preincubation. The conversion of α-ketoisovalerate was monitored directly at 314 nm. Experimental data were fitted according to equation $v_{obs} = V_{max}S/(A + B\cdot S + S^2)$. $E$, comparison of $k_{obs}$ values for amino acids and their corresponding α-keto acids (closed squares, L-isoleucine; open squares, α-keto-β-methylvalerate; closed circles, L-alanine; open circles, pyruvate). Experimental data were fitted according to Equation 3 for α-keto acid and to Equation 16 for amino acid dependences.
Amino Acids as Allosteric Regulators of Enzyme Activity

ThDP and 2-hydroxyethyl-ThDP is altered, implying that also the rate ratio of decarboxylation and aldehyde release is slightly modified by this activator.

**Correlation between the Kinetic Model and the Experimental Data**—In the absence of amino acids the model concomitantly explains (i) the hyperbolic v/S-plots of the initial velocities (v<sub>0</sub>, Equation 4), (ii) the sigmoid v/S-plots of the velocities in the presence of 40 mM benzoylformate; fits are according to Equation 16.

**TABLE 3**

Microscopic constants for the activation of MtKDC by amino acids

| Amino acid | k<sup>a</sup> | k<sub>max</sub> | k<sub>max</sub>/k<sub>obs</sub> | k<sub>max</sub>/k<sub>obs</sub> |
|------------|---------------|----------------|-------------------|----------------|
| L-Leu      | 0.021 ± 0.012 | 0.225 ± 0.006 | 1.53 ± 0.28       | 0.147          |
| L-Ile      | 0.022 ± 0.013 | 0.258 ± 0.010 | 1.74 ± 0.34       | 0.148          |
| L-Val      | 0.018 ± 0.016 | 0.258 ± 0.021 | 4.34 ± 1.28       | 0.059          |
| L-Phe      | 0.014 ± 0.007 | 0.262 ± 0.056 | 3.80 ± 0.45       | 0.069          |
| D-Val      | 0.016 ± 0.002 | 0.205 ± 0.008 | 119.3 ± 11.8      | 0.002          |
| D-Phe<sup>a</sup> | 0.019 ± 0.001 |               |                   | 0.001          |
| L-Ala      | 0.013 ± 0.002 | 0.124 ± 0.007 | 157.8 ± 16.8      | 0.0008         |

*Because of insufficient solubility, no saturation for the activation rate constant could be reached. Therefore, the k<sub>max</sub> and k<sub>obs</sub> values could not be determined. For L-tryptophan, only k<sup>a</sup> was determined (0.017 ± 0.0004 s<sup>-1</sup>). The activation potential of amino acids is given by the ratio of k<sub>max</sub>/k<sub>obs</sub>.*

**FIGURE 5.** Dependence of the observed rate constant of activation k<sub>obs</sub> on the amino acid concentration and substrate concentration, respectively. A, dependence of k<sub>obs</sub> on the amino acid concentration (L-leucine) for two substrate concentrations (closed inverted triangles, 3 mM benzoylformate; closed triangles, 40 mM benzoylformate); fits are according to Equation 16. B, dependence of the dissociation constant K<sub>obs</sub> of L-leucine on benzoylformate concentration. Experimental data were fitted according to Equation 19. C, dependence of k<sub>obs</sub> on the substrate concentration of benzoylformate in the absence (open circles) and the presence of 40 mM benzoylformate (open squares). Experimental data in the absence and the presence of L-leucine were fitted according to Equation 3 and Equation 16, respectively. D, three-dimensional plot of the dependence of the activation rate constant k<sub>obs</sub> on substrate and amino acid concentration, given as superposition of A and C.
development of complex activation models necessary (32, 33). In the case of MtKDC, all transients are perfectly single exponential under all conditions applied. This has been checked by comprehensive analyses of residual plots (data not shown). However, we cannot exclude that hidden kinetic complexity might surface under different experimental conditions, e.g., at other pH values.

Conclusions—Differences in the allosteric regulation mechanism of MtKDC and substrate-activated PDCs described above may be attributed to the fact that those enzymes are important in different metabolic pathways. The substrate spectrum of MtKDC presumably requires an enhanced degree of the active center hydrophobicity as compared with that of the active centers of PDCs. Strong hydrophobicity and/or low polarity of the cofactor environment is paramount to fast H/D exchange at the cofactor C2 atom (55–57). Therefore, in a strongly hydrophobic active center deprotonation at C2 is rapid even before activation. Conclusively, another step has to take the role of the target of activation (i.e., C-C-bond formation in case of MtKDC). Whereas PDCs are key enzymes in the alcoholic fermentation, ARO10, KdcA, and MtKDC play major roles in the amino acid degradation. Thus, it is not surprising that the substrate-activated ScPDC is not modulated by amino acids (analyzed for concentrations up to 750 mM l-alanine; data not shown).

For the ThDP-dependent decarboxylases participating in either the alcoholic fermentation or the amino acid degradation, enzymes were described that either display or lack allosteric regulation. ScPDC and KIPDC are both allosterically regulated by their substrate pyruvate. Examples for non-regulated ThDP-dependent enzymes involved in amino acid degradation are EelPDC (40) and KdcA (7). The mycobacterial enzyme investigated in this study and the phenylpyruvate decarboxylase from Azospirillum brasilense (53) are examples for allosterically regulated enzymes involved in amino acid catabolism.

MtKDC. For further studies on the mechanism of amino acid activation, kinetic and structural investigations of ARO10 and phenylpyruvate decarboxylase from A. brasilense (53) might be interesting. Particularly, structural data are necessary to obtain more information on activator binding. Small angle x-ray scattering experiments with MtKDC demonstrated structural rearrangements upon the addition of amino acids (43). Hitherto, attempts to crystallize MtKDC have been unsuccessful.

Transposon site hybridization experiments showed that MtKDC expression is not essential for the optimal growth of Mycobacterium tuberculosis (38). Thus, MtKDC is not a potential target for novel anti-tuberculosis drugs. However, our findings might be of common interest for studies in the field of metabolic pathway regulation, enzyme catalysis, and more specifically, regulation of ThDP-dependent enzymes.

Acknowledgments—We thank Angelika Schierhorn for performing mass spectroscopy measurements and Manfred S. Weiss for material support for MtKDC cloning.

REFERENCES

1. Schellenberger, A. (1998) Biochim. Biophys. Acta 1385, 177–186
2. Neuberg, C., and Karczag, L. (1911) Biochem. Z. 36, 68–75
3. Hegeman, G. D. (1966) J. Bacteriol. 91, 1140–1154
4. Weiss, P. M., Garcia, G. A., Kenyon, G. L., Cleland, W. W., and Cook, P. F. (1988) Biochemistry 27, 2197–2205
5. Koga, J., Adachi, T., and Hidaka, H. (1992) J. Biol. Chem. 267, 15823–15828
6. Somers, E., Ptcacek, D., Gyssegom, P., Srinivasan, M., and Vanderleyden, J. (2005) Appl. Environ. Microbiol. 71, 1803–1810
7. Yep, A., Kenyon, G. L., and McLeish, M. J. (2006) Biog. Chem. 34, 325–336
8. Smit, B. A., van Hylckama Vlieg, J. E. T., Engels, W. J., Meijer, L., Wouters, J. T., and Smit, G. (2005) Appl. Environ. Microbiol. 71, 303–311
9. Vuralhan, Z., Luttik, M. A., Tai, S. L., Boer, V. M., Morais, M. A., Schipper, D., Almering, M. J., Kotter, P., Dickinson, J. R., Daran, J. M., and Pronk, J. T. (2005) Appl. Environ. Microbiol. 71, 3276–3284
Amino Acids as Allosteric Regulators of Enzyme Activity

10. Dickinson, J. R., Salgado, L. E., and Hewlins, M. J. (2003) J. Biol. Chem. 278, 8028–8034
11. Vuralhan, Z., Morais, M. A., Tai, S. L., Piper, M. D., and Pronk, J. T. (2003) Appl. Environ. Microbiol. 69, 4534–4541
12. Breunig, K. D., Bolotin-Fukuhara, M., Bianchi, M. M., Bourgarel, D., Falcone, C., Ferrero, I. I., Frontali, L., Goffrini, P., Krüger, J. J., Mazzoni, C., Milkowski, C., Steensma, H. Y., Wesolowski-Louvel, M., and Zeeman, A. M. (2000) Enzyme Microb. Technol. 26, 771–780
13. Kiers, J., Zeeman, A. M., Luttkink, M., Thiele, C., Castrillo, J. I., Steensma, H. Y., van Dijken, J. P., and Pronk, J. T. (1998) Eur. J. Biochem. 259, 3256–3263
14. Krieger, F., Spinka, M., Golbik, R., Hübner, G., and König, S. (2002) Eur. J. Biochem. 269, 3256–3263
15. Hübner, G., Weidhase, R., and Schellenberger, A. (1978) Eur. J. Biochem. 92, 175–181
16. Dietrich, A., and König, S. (1997) FEBS Lett. 400, 42–44
17. Davies, D. D. (1967) Proc. Biochem. Soc. 104, 50P
18. Zehender, H., Trescher, D., and Ullrich, J. (1987) Eur. J. Biochem. 167, 149–154
19. Neuser, F., Zorn, H., Richter, U., and Berger, R. G. (2000) Biol. Chem. 381, 349–353
20. Acar, S., Yücel, M., and Hamamci, H. (2007) Enzyme Microb. Technol. 40, 675–682
21. Alvarez, F. J., Ermer, J., Hübner, G., Schellenberger, A., and Schwen, R. L. (1991) J. Am. Chem. Soc. 113, 8402–8409
22. Dyda, F., Furey, W., Swaminathan, S., Sax, M., Farrenkopf, B., and Jordan, F. (1993) Biochemistry 32, 6165–6170
23. Arjunan, P., Umland, T., Dyda, F., Swaminathan, S., Furey, W., Sax, M., Farrenkopf, B., Gao, Y., Zhang, D., and Jordan, F. (1996) J. Mol. Biol. 256, 590–600
24. Furey, W., Arjunan, P., Chen, L., Sax, M., Guo, F., and Jordan, F. (1998) Biochim. Biophys. Acta 1385, 253–270
25. Lu, G., Dobritsch, D., König, S., and Schneider, G. (1997) FEBS Lett. 403, 249–253
26. Lu, G., Dobritsch, D., Baumann, S., Schneider, G., and König, S. (2000) Eur. J. Biochem. 267, 861–868
27. Baburina, I., Dikdan, G., Guo, F., Tous, G. I., Root, B., and Jordan, F. (1998) Biochemistry 37, 1245–1255
28. Baburina, I., Li, H., Bennion, B., Furey, W., and Jordan, F. (1998) Biochemistry 37, 1235–1244
29. Li, H., and Jordan, F. (1999) Biochemistry 38, 10004–10012
30. Li, H., Furey, W., and Jordan, F. (1999) Biochemistry 38, 9992–10003
31. Joseph, E., Wei, W., Tittmann, K., and Jordan, F. (2006) Biochemistry 45, 3517–13527
32. Sergienko, E. A., and Jordan, F. (2001) Biochemistry 40, 7382–7403
33. Sergienko, E. A., and Jordan, F. (2002) Biochemistry 41, 3952–3967
34. Schwen, R. L. (2007) Isotopes Environ. Health Stud. 43, 1–16
35. Fischer, G. (1971) Investigations on Structure and Reactivity of Alpha-keto Acids and Alpha-keto Amides. Doctoral dissertation, Martin-Luther University Halle-Wittenberg
36. Rudolph, R., Böhm, G., Lilie, H., and Jänicke, R. Folding proteins (1997) in Protein Function, a Practical Approach (Creighton, T. E., ed) pp. 55–99, IRL Press at Oxford University Press, Oxford
37. Bradford, M. M. (1976)
38. Sassetti, C. M., Boyd, D. H., and Rubin, E. J. (2003) Mol. Microbiol. 48, 77–84
39. Holzer, H., Schultz, G., Villar-Palasi, C., and Jüntgen-Sell, J. (1956) Biochem. Z. 327, 331–344
40. Schütz, A., Golbik, R., Tittmann, K., Svergun, D. I., Koch, M. H. J., Hübner, G., and König, S. (2003) Eur. J. Biochem. 270, 2322–2331
41. Kern, D., Kern, G., Neef, H., Tittmann, K., Killenberg-Jabs, M., Wickner, C., Schneider, G., and Hübner, G. (1997) Science 275, 67–70
42. Tittmann, K., Golbik, R., Uhlmann, K., Khazlova, L., Schneider, G., Patel, M., Jordan, F., Chipman, D. M., Duggleby, R. G., and Hübner, G. (2003) Biochemistry 42, 7885–7891
43. Werther, T., Konarev, P., Svergun, D. I., and König, S. (2006) Hasylab Annual Report, pp. 361–362, Hamburger Synchrotronstrahlungslabor HASYLAB am Deutschen Elektronen-Synchrotron DESY in der Helmholtz-Gemeinschaft HGF, Hamburg, Germany
44. Lehmén, H., Fischer, G., Hübner, G., Kohnert, K. D., and Schellenberger, A. (1973) Eur. J. Biochem. 32, 83–87
45. Bringer-Meyer, S., Schimz, K. L., and Sahm, H. (1986) Arch Microbiol. 146, 105–110
46. Hübner, G., and Schellenberger, A. (1986) Biochem. Int. 13, 767–772
47. Baburina, I., Gao, Y., Hu, Z., Jordan, F., Hohmann, S., and Furey, W. (1994) Biochimica et Biophysica Acta 1249, 1–13
48. Wang, J., Golbik, R., Seliger, B., Spinka, M., Tittmann, K., Hübner, G., and Jordan, F. (2001) Biochemistry 40, 1755–1763
49. Frank, R. A. W., Titman, C. M., Pratap, J. V., Luisi, B. F., and Perham, R. N. (2004) Science 306, 872–876
50. Jordan, F., Nemeria, N. S., and Sergienko, E. (2005) Acc. Chem. Res. 38, 755–763
51. Versées, W., Spaepen, S., Vanderleyden, J., and Steyaert, J. (2007) FEBS J. 274, 2363–2375
52. Versées, W., Spaepen, S., Wood, M. D. H., Leeper, F. J., Vanderleyden, J., and Steyaert, J. (2007) J. Biol. Chem. 282, 35269–35278
53. Crosby, J., and Lienhard, G. E. (1970) J. Am. Chem. Soc. 92, 5707–5716
54. Jordan, F., Li, H., and Brown, A. (1999) Biochemistry 38, 6369–6373
55. Zhang, S., Zhou, L., Nemeria, N., Yan, Y., Zhang, Z., Zou, Y., and Jordan, F. (2005) Biochemistry 44, 2237–2243