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Studying the Lysine Acetylation of Malate Dehydrogenase

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Abstract

Protein acetylation plays important roles in many biological processes. Malate dehydrogenase (MDH), a key enzyme in the tricarboxylic acid (TCA) cycle, has been identified to be acetylated in bacteria by proteomic studies, but no further characterization has been reported. One challenge for studying protein acetylation is to get purely acetylated proteins at specific positions. Here we applied the genetic code expansion strategy to site-specifically incorporate N^ε-acetyllysine into MDH. The acetylation of lysine residues in MDH could enhance its enzyme activity. The *Escherichia coli* deacetylase CobB could deacetylate acetylated MDH, while the *E. coli* acetyltransferase YfiQ cannot acetylate MDH efficiently. Our results also demonstrated that acetyl-CoA or acetyl-phosphate could acetylate MDH chemically *in vitro*. Furthermore, the acetylation level of MDH was shown to be affected by carbon sources in the growth medium.

Graphical abstract

Keywords

post-translational modification; acetyltransferase; deacetylase; genetic code expansion; protein acetylation

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INTRODUCTION

The acetylation of lysine residues is one of the most common post-translational modifications of proteins that regulates diverse cellular functions including DNA-protein interaction, transcription activity, protein stability, stress response, apoptosis, cellular differentiation, and energy metabolism [1–13]. The abnormality of acetylation modifications is associated with diabetes, cardiovascular diseases, cancers, and neurodegenerative disorders [14–24]. During the last decade, proteomic studies have identified lysine acetylation in thousands of eukaryotic proteins which significantly expanded our knowledge of protein acetylation [25–27].

Interestingly, protein acetylation in bacteria starts to attract attentions in the last five years [28–35]. A series of proteomic studies have identified hundreds of bacterial proteins with lysine acetylation, including metabolic enzymes, stress response proteins, regulator of chemotaxis, chaperones, as well as transcription and translation factors [36–47]. However, few studies have been performed to further characterize these acetylated proteins. Take *E. coli* as an example, only six proteins with lysine acetylation were studied, including acetyl-CoA synthetase [48–51]; CheY, the regulator of bacterial chemotaxis [52–54]; RcsB, the regulator of capsule synthesis [55–57]; RNase R [58], *N*-hydroxyarylamine *O*-acetyltransferase [59], and α -subunit of RNA polymerase [60, 61]. One challenge for studying lysine acetylation is that it is difficult to synthesize purely acetylated proteins at specific sites by most classic methods. To solve this problem, the genetic code expansion strategy has been applied. Rather than adding acetyl group after protein translation, this approach uses an orthogonal pair of an engineered pyrrolysyl-tRNA synthetase variant and its cognate tRNA from *Methanosarcinaceae* species to co-translationally direct the incorporation of N^e-acetyllysine (AcK) in response to a stop codon at desired positions in target proteins [62–65].

MDH, a widely distributed enzyme catalyzing the conversion of oxaloacetate and malate, plays key roles in many important metabolic pathways including the tricarboxylic acid (TCA) cycle, glyoxylate bypass, amino acid synthesis, gluconeogenesis, and the exchange of metabolites between cytoplasm and subcellular organelles [66, 67]. Previous studies have shown that the acetylation of lysine residues in mammalian MDHs is involved in the cross-talk mechanisms between adipogenesis and the intracellular energy level [68–70]. However, the acetylation of bacterial MDH has not been characterized before. Here, we applied the genetic code expansion strategy to study the lysine acetylation of MDH in *E. coli*. We also used the same strategy to study human MDH as comparison.

RESULTS

Selecting acetylation sites in MDHs

Recently, several proteomic studies have identified lysine acetylation in *E. coli* MDH (eMDH), and lysine residues K99 and K140 were identified to be acetylated in all of these reports [37–39, 46, 47]. So we chose these two positions to incorporate AcK. In human cells, there are two MDH isozymes, the cytosol MDH1 and mitochondrial MDH2 [71]. Human MDH2 (hMDH2) has higher homology to eMDH than hMDH1 does [72]. Previous

proteomic studies showed that hMDH2 has four lysine residues acetylated at positions K185, K301, K307, and K314 [73]. So we also chose these four positions to incorporate AcK as comparison. Interestingly, the alignment of eMDH and hMDH2 showed different patterns: the acetylation sites in eMDH are at the middle part of the primary sequence, while those in hMDH2 are mainly at the C-terminus (Figure S1). The acetylation site K185 in hMDH2 has its counterpart in eMDH at position K162 which was also selected to incorporate AcK as a control.

Site-specifically incorporating lysine acetylation

We incorporated AcK at selected positions mentioned above in both eMDH and hMDH2 by our recently optimized AcK incorporation system which has an optimized tRNA^{Pyl} with better binding with *E. coli* elongation factor (EF-Tu), thus increasing the incorporation efficiency [65]. The AcK was genetically encoded by an amber stop codon (TAG) which was introduced by site-directed mutagenesis. Previous studies showed that the K12-derived strains have substantially higher acetylation than B-strain-derived BL21 cells during growth [39]. To lower the background of non-specific acetylation at other lysine residues, we used BL21 (DE3) strain as the expression host. The incorporation of AcK was confirmed by both western blotting (Figure 1A) and mass spectrometry (MS) (Figure S2–S8).

The lysine acetylation of MDH increases its enzyme activities

The enzyme activities of eMDH and hMDH2 as well as their acetylated variants were measured (Figure 1B). For eMDH, the acetylation at positions K99 and K140 increased the enzyme activity by 2.3 and 3.4 folds, individually, which is consistent with our previous study [65], while the acetylation at the position K162 had little effect. Our previous study also showed that doubly acetylated eMDH at both positions K99 and K140 had 6-fold higher enzyme activity than that of wild-type eMDH [65]. For hMDH2, only the acetylation at the position K307 increased the enzyme activity by 3.9 folds, while others had no obvious effects.

Steady-state kinetic analyses were performed with wild-type MDHs and their acetylated variants (Table 1). The lysine acetylation appeared not to affect the K_M values of both substrates, NAD⁺ and malate, indicating that the increase of enzyme activities results mainly from the improvement of the overall turnover.

CobB can deacetylate acetylated MDHs at specific positions

Lysine deacetylases remove the N-acetyl amide moieties, and can be broadly divided into two families according to their reaction mechanism: hydrolytic deacetylases and NAD⁺-dependent deacetylases which are also named as sirtuins [32, 74]. The CobB protein, a sirtuin family member, was found in many bacteria including *E. coli* and *Salmonella* [48, 51]. Recently, YcgC has been confirmed to be a hydrolytic deacetylase and target a distinct set of substrates from *E. coli* CobB, representing a novel family of prokaryotic deacetylases [75].

To determine the deacetylation activity of the CobB protein on acetylated MDHs, K12-derived *E. coli* TOP10 strain with higher acetylation levels was used for *in vivo* tests [39].

The genes of eMDH and hMDH2 with C-terminal His₆ tags were expressed and purified in wild-type or *cobB* cells, individually. Western blotting was used for detecting the acetylation (Figure 2A). The deletion of *cobB* gene increased the acetylation levels of both eMDH and hMDH2, indicating that CobB could deacetylate their lysine acetylation *in vivo*. We also measured the enzyme activities of MDHs from wild-type or *cobB* cells (Figure 2B). The MDHs purified from *cobB* cells had higher enzyme activities, which was consistent with the western blotting results as lysine acetylation could increase MDH activities.

We also performed *in vitro* deacetylation experiments. The optimized AcK incorporation system mentioned above was used to generate site-specifically acetylated eMDH at positions K99, K140, and K162, individually, and acetylated hMDH2s at positions K185, K301, K307, and K314, respectively. All these MDH variants were treated with CobB protein separately. Western blotting was used for detecting the acetylation (Figure 2C). Our results showed that CobB was specific for positions K140 and K162 of eMDH and the position K307 of hMDH2. After 2-hour treatment of CobB, no detectable acetylation was observed for these three positions by western blotting, while the acetylation at other positions were not changed obviously. The insensitivity of acetyl-K99 in eMDH against CobB supports the conclusion from previous studies that although CobB is the predominate deacetylase in *E. coli*, it could not deacetylate the majority of acetylation at lysine residues [39, 46, 76]. For hMDH2 acetylated variants, we also tested them with the counterpart of CobB in human, SIRT3, which is the major protein deacetylase in mitochondria [77]. Similarly, SIRT3 is only specific for acetyl-K307 of hMDH2 (Figure S9).

The acetylation of MDHs

The acetylation of lysine residues is catalyzed by acetyltransferases which can be categorized into five families: the Gcn5-related N-acetyltransferase (GNAT) family, the MYST family, the CBP/p300 co-activators, the SRC family of co-activators, and the TAFII group of transcription factors [78–81]. YfiQ, a member of GNAT family, is the only known acetyltransferase in *E. coli*, required for glucose-dependent acetylation of several lysine residues within RNA polymerase [58] and for the acetylation of Lys544 in RNase R [61].

To determine the acetylation activity of the YfiQ protein on eMDH, TOP10 strain (a K12-derived strain with a higher acetylation level) was used for *in vivo* tests. The gene of eMDH with a C-terminal His₆ tag was expressed in TOP10 *cobB yfiQ* cells and purified. Western blotting was used for detecting the acetylation. Compared with the eMDH which was expressed from TOP10 *cobB* cells (Figure 2A), the deletion of *yfiQ* gene did not decrease the acetylation level of eMDH with the *cobB* background, indicating that the YfiQ protein is not the major acetyltransferase for eMDH *in vivo*. We also measured the enzyme activity of eMDH from TOP10 *cobB yfiQ* cells (Figure 3A). eMDH purified from *cobB YfiQ* cells had a similar enzyme activity with eMDH purified from *cobB* cells, indicating they had similar levels of acetylation which was consistent with the western blotting results.

We also performed *in vitro* acetylation experiments. The wild-type eMDH expressed and purified from BL21 (DE3) cells which had no detectable acetylation with western blotting

(Figure 1B) was treated with purified YfiQ protein and the acetylation donor, acetyl-CoA. Incubated with YfiQ and acetyl-CoA for 2 hours, the acetylation level of eMDH had no obvious difference with that incubated with acetyl-CoA only, indicating that YfiQ cannot acetylate eMDH efficiently *in vitro* (Figure 3B).

Besides YfiQ, there are 24 members of GNAT family acetyltransferases in *E. coli*, 12 with known substrates: ArgA, AstA, CitC, MnaT, PanM, PhnO, RimI, RimJ, RimL, SpeG, TmcA, and WecD; the other 12 proteins with unknown functions: ElaA, YafP, YedL, YhbS, YhhY, YiaC, YiiD, YjaB, YjdJ, YjgM, YjhQ, and YpeA [33]. We tested their functions in acetylating eMDH *in vitro*. Similarly, incubated with individual candidate acetyltransferase and acetyl-CoA for 2 hours, the acetylation level of eMDH had no obvious difference with that incubated with acetyl-CoA only (Figure S10).

Previous studies showed chemical acetylation of proteins both *in vitro* and *in vivo* [39, 46, 82–85]. We also noticed that eMDH was acetylated after incubation with only acetyl-CoA for 2 hours (Figure 3B). So we determined the chemical acetylation of eMDH by acetyl-CoA or acetyl-phosphate, respectively. eMDH expressed and purified from BL21(DE3) cells which has no detectable acetylation was used. Western blotting was used to detect the acetylation (Figure 3C). The results showed that both acetyl-CoA and acetyl-phosphate could chemically acetylate eMDH at a dose-dependent manner. And the acetylation level of eMDH increased with the incubation time, which is consistent with the previous study on protein acetylation dynamics [47]. Such acetylation accumulation may also result from a carbon-to-nitrogen or a carbon-to-magnesium imbalance *in vivo* [39, 86].

Acetylation of eMDH with different carbon sources

Previous studies showed that the acetylation level of metabolic enzymes is higher when cells grow with glucose [73]. So we determined the acetylation level of eMDH purified from TOP10 cells grown with different carbon sources. Clearly, glucose could increase the acetylation level of eMDH with a dose-dependent manner (Figure 4A). We also measured the enzyme activities of eMDH from cells with different carbon sources, and it was consistent with the western blotting results (Figure 4B). These results are consistent with previous proteomic studies [47], indicating the possible role of enzyme acetylation in mediating cellular adaptation to different environments.

DISCUSSION

Classic approaches use amino acid substitutions to map functional lysine acetylation sites. The substitution with arginine retains a positive charge which is often utilized as a non-acetylated mimic, while the substitution with glutamine abolishes the positive charge which can act as a surrogate of acetylation [87–89]. However, such strategies sometimes do not reveal the real effects of lysine acetylation [90]. Here, we synthesized homogeneously acetylated proteins at specific sites by the genetic code expansion strategy to determine the effects of acetylation directly, overcoming the potential problems with the substitution approach.

Our kinetic analysis of MDH variants indicated that the catalytic efficiency of acetylated MDHs increased 2.3 to 3.4 folds (Table 1). The K_M values of both substrates, NAD^+ and malate, did not change obviously, indicating that acetylation at these positions do not affect the substrate binding. The results also showed that the human enzyme has higher catalytic efficiency and much better binding of malate than those of the *E. coli* enzyme.

Based on the crystal structures on eMDH (PDB ID: 1EMD) and hMDH2 (PDB ID: 2DFD) [91], we mapped the selected acetylated residues used in this study (Figure 5). None of these acetylated sites are at substrate binding sites. It is consistent with our kinetic analyses which indicated that the lysine acetylation does not affect the substrates binding (Table 1). Our results also showed that CobB deacetylase was specific for positions K140 and K162 of eMDH and the position K307 of hMDH2 (Figure 2C). From the structures of MDHs (Figure 5), we found that these three residues are located at coiled structures. Lysine residues at position K99 in eMDH, positions K301 and K314 in hMDH2 are within either helical structures or sheet structures. These results suggested CobB favors unstructured protein domains which is consistent with previous studies [39, 46, 76, 92]. Interestingly, as a counterpart of eMDH position K162, the lysine acetylation at position K185 (at a coiled structure) in hMDH2 is resistant to the deacetylase (Figure 2C), indicating that the specificity of the deacetylase may also depend on primary sequence contexts, which has been proposed in the previous study [76]. And this difference may be an ideal target for antibacterial agent development.

Based on the bioinformatical analyses, there are 25 putative GNAT family acetyltransferases in *E. coli* [78]. Some members perform $N\alpha$ -acetylation such as RimL [93], while some members are specific for free amino acids like ArgA [94]. There are reports to describe the functions of only 13 GNAT members, leaving the other 12 members completely unknown [95]. Although some works have been done to identify the protein motifs which are recognized by protein acetyltransferases [95, 96], the difference between GNAT members may be important for their specificities for different protein substrates, thus it is necessary to characterize those 25 GNAT members for protein acetylation studies as we did in this study (Figure S10).

MATERIALS AND METHODS

General molecular biology

The amino acids in this study were purchased from Sigma-Aldrich or ChemImpex. TOP10 cells (Life Technologies) were used for general cloning. All the cloning experiments were performed by using the Gibson Assembly kit (New England Biolabs). The mutations of stop codons in MDH genes were made by the QuikChange II mutagenesis kit (Agilent Technologies). The strains and plasmids used in this study is listed in Table S1. *Western Blots*: The purified MDHs and their variants were fractionated by SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad). The membrane was incubated at room temperature with gentle shaking in TTBS and 5% Milk blocking buffer for 60 min. The primary antibody, HRP-conjugated-Acetylated-Lysine (Ac-K²-100) Rabbit mAb (Cell Signaling Technology), was diluted 1:1000 and soaked the membranes overnight at 4 degree. The membrane was prepared for detection using Western Lighting Plus-ECL (PerkinElmer,

Inc.). The *E. coli* proteins including CobB and 25 GNAT family acetyltransferases were purified from the ASKA strain collection by following the purification protocols [97]. The human SIRT3 was purchased from Sigma-Aldrich (St. Louis, MO, USA). The gene deletion in *E. coli* was performed by recombination as previous protocols [98].

Expression and purification of AcK-containing MDH variants

The genes of MDHs and their variants were cloned into the *pET15b* or *pBAD* plasmid with a C-terminal His₆-tag and transformed into BL21 (DE3) or Top 10 cells together with the *pTech* plasmids harboring the AcK incorporation system for expression. For human MDH2 expression, the predicted mitochondrial targeting sequence (residues 2–24) was removed. The expression strain was grown on 1 L of LB medium supplemented with 100 µg/ml ampicillin and 50 µg/ml chloramphenicol at 37°C to an absorbance of 0.6–0.8 at 600 nm and protein expression was induced by the addition of 1 mM IPTG and supplemented with 5 mM AcK and 20 mM nicotinamine (NAM, deacetylases inhibitor). Cells were incubated at 30°C for an additional 8 h and harvested by centrifugation at 5000 × g for 10 min at 4 °C. The cell paste was suspended in 15 ml of lysis buffer (50 mM Tris (pH 7.5), 300 mM NaCl, 20 mM imidazole, 20 mM NAM) and broken by sonication. The crude extract was centrifuged at 20,000 × g for 30 min at 4°C. The soluble fraction was loaded onto a column containing 2 ml of Ni-NTA resin (Qiagen) previously equilibrated with 20 ml lysis buffer. The column was washed with 50 ml lysis buffer. The protein bound to the column was then eluted with 2 ml of 50 mM Tris (pH 7.5), 300 mM NaCl, 150 mM imidazole. The purified protein was dialyzed with 50 mM Tris (pH 7.5), 50 mM NaCl, 1mM DTT and 50% glycerol, and stored at –80°C for further studies.

MDH enzyme assays

The assays were performed by following the instruction of the EnzyChrom™ Malate Dehydrogenase Assay Kit (EMDH-100) from BioAssay Systems. This non-radioactive, colorimetric MDH assay is based on the reduction of the tetrazolium salt MTT in a NADH-coupled enzymatic reaction to a reduced form of MTT which exhibits an absorption maximum at 565 nm. The increase in absorbance at 565 nm is proportional to the enzyme activity.

LC-MS/MS analyses

The proteins were trypsin digested by a standard in-gel digestion protocol, and analyzed by LC-MS/MS on an LTQ Orbitrap XL (Thermo Scientific) equipped with a nanoACQUITY UPLC system (Waters). A Symmetry C18 trap column (180 µm × 20 mm; Waters) and a nanoACQUITY UPLC column (1.7 µm, 100 µm × 250 mm, 35°C) were used for peptide separation. Trapping was done at 15 µL min^{–1}, 99% buffer A (water with 0.1% formic acid) for 1 min. Peptide separation was performed at 300 nL min^{–1} with buffer A and buffer B (CH₃CN containing 0.1% formic acid). The linear gradient (51 min) was from 5% buffer B to 50% B at 50 min, to 85% B at 51 min. MS data were acquired in the Orbitrap with one microscan, and a maximum inject time of 900 ms followed by data-dependent MS/MS acquisitions in the ion trap (through collision induced dissociation, CID). The Mascot search algorithm was used to search for the appropriate noncanonical substitution (Matrix Science, Boston, MA).

In vitro acetylation

The reaction was performed in the buffer containing 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 10% glycerol, 1 mM DTT and 10 mM sodium butyrate. The acetylation was carried out by adding 10 µg MDHs, 10 µg of candidate acetyltransferase, and 0.2 mM acetyl-CoA in a volume of 100 µl. Reaction mixtures were completely mixed and incubated at 37 °C.

CobB-mediated in vitro deacetylation

The reaction was performed in buffer contains 40 mM HEPES (pH 7.0), 6 mM MgCl₂, 1.0 mM NAD⁺, 1 mM DTT and 10 % glycerol. 10 µg MDHs, 10 µg CobB, and the reaction buffer were incubated at 37 °C in a total volume of 100 µl.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AcK	N ^ε -acetyllysine
MDH	malate dehydrogenase
eMDH	<i>Escherichia coli</i> malate dehydrogenase
hMDH2	human malate dehydrogenase isozyme 2
TCA	tricarboxylic acid
MS	mass spectrometry
GNAT	Gcn5-related N-acetyltransferase
NAM	nicotinamine

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Highlights

- Acetyllysine was site-specifically incorporated into the malate dehydrogenase.
- The acetylation of malate dehydrogenase enhanced enzyme activity.
- CobB could deacetylate acetylated malate dehydrogenase.
- Malate dehydrogenase could be acetylated chemically.
- The acetylation level of malate dehydrogenase was affected by carbon sources.

Figure 1. AcK incorporation into MDHs

A) SDS-PAGE and western blotting analyses of purified MDHs and their variants. Lane 1, wild-type eMDH; lane 2, eMDH 99AcK; lane 3, eMDH 140AcK; lane 4, eMDH 162AcK; lane 5; wild-type hMDH2; lane 6, hMDH2 185 AcK; lane 7, hMDH2 301AcK; lane 8, hMDH2 307AcK; lane 9, hMDH2 314AcK. The same amounts of proteins were loaded. B) The enzyme activities of MDHs and their acetylated variants. The activities of wild-type *E. coli* MDH and human MDH2 were set as 1, respectively. The mean values and standard errors were calculated based on three replicates.

Figure 2. CobB deacetylates MDHs

A) Western blotting of purified MDHs from TOP10 and TOP10 *cobB* cells. Lane 1 and 4 were from wild-type TOP10 cells. Lane 2 and 5 were from TOP10 *cobB* cells. Lane 3 was from TOP10 *cobB yfiQ* cells. The same amounts of proteins were loaded. B) The enzyme activities of purified MDHs from TOP10 and TOP10 *cobB* cells. The activity of eMDH purified from wild-type TOP10 cells was set as 1. The mean values and standard errors were calculated based on three replicates. C) Western blotting of acetylated MDH variants treated with the CobB protein. The acetylation levels of MDH variants after 2-hour incubation were compared with those without CobB treatment. The same amounts of proteins were loaded.

Figure 3. Acetylation of *E. coli* MDH

A) The enzyme activities of purified eMDHs from TOP10, TOP10 *cobB*, and TOP10 *cobB yfiQ* cells. The activity of eMDH purified from TOP10 cells was set as 1. The mean values and standard errors were calculated based on three replicates. B) Western blotting of purified eMDH from BL21(DE3) treated with the YfiQ protein and acetyl-CoA for 2 hours. The same amounts of proteins were loaded. C) B) Western blotting of purified eMDH from BL21(DE3) treated with acetyl-CoA (AcCoA) or acetyl-phosphate (AcP) for 2 hours and 12 hours, respectively. The same amounts of proteins were loaded.

Figure 4. Acetylation of *E. coli* MDH with different carbon sources

A) Western blotting of purified eMDHs from TOP10 cells grown with different carbon sources. LB medium, minimal medium with different glucose (Glu) concentrations (5%, 2%, 1%, or 0.5%) were used. The same amounts of proteins were loaded. B) The enzyme activities of purified eMDHs from TOP10 cells grown with different carbon sources. The activity of eMDH purified from TOP10 cells grown with LB medium was set as 1. The mean values and standard errors were calculated based on three replicates.

Figure 5. Mapping of acetylation sites on the crystal structures of MDHs
The crystal structures of eMDH (PDB ID: 1EMD) and hMDH2 (PDB ID: 2DFD) were demonstrated with the selected acetylation sites in this study labelled.

Table 1Kinetic analyses of MDHs and acetylated variants^a

	k_{cat} (s ⁻¹)	K_M, NAD^+ (mM)	$K_M, malate$ (mM)	$k_{cat}/K_M, NAD^+$ (S ⁻¹ mM ⁻¹)	$k_{cat}/K_M, malate$ (S ⁻¹ mM ⁻¹)
eMDH WT	20.2 ± 0.4	0.23 ± 0.02	2.61 ± 0.20	87.8	7.74
eMDH 99AcK	51.2 ± 1.3	0.25 ± 0.07	2.32 ± 0.32	204.8	22.07
eMDH 140AcK	74.3 ± 1.1	0.26 ± 0.06	2.54 ± 0.64	285.8	29.25
hMDH2 WT	69.6 ± 2.9	0.16 ± 0.01	0.24 ± 0.08	435.0	290.0
hMDH2 307AcK	178.9 ± 3.1	0.12 ± 0.04	0.20 ± 0.12	1490.8	894.5

^aThe mean values and standard errors were calculated based on three replicates. The parameters were determined by nonlinear regression with software GraFit (Eriihacus Software).