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Observation of a chemically labile, noncovalent enzyme intermediate in the reaction of metal-dependent *Aquifex pyrophilus* KDO8PS by time-resolved mass spectrometry

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Abstract

The direct detection of intermediates in enzymatic reactions can yield important mechanistic insights but may be difficult due to short intermediate lifetimes and chemical instability. Using a rapid-mixing device coupled with electrospray ionization time-of-flight mass spectrometry, the noncova-lent hemiketal intermediate in the reaction of metal-dependent 3-deoxy-D-manno-octulosonate-8-phosphate (KDO8P) synthase from *Aquifex pyrophilus* was observed in the millisecond time range. Using single turnover conditions, the noncovalent complexes of enzyme with Cd^{2R}:phosphoenolpyruvate, Cd^{2R}:phosphate, Cd^{2R}:KDO8P, and Cd^{2R}:intermediate complexes were resolved. The intermediate complex is present during times ranging from 50–630 ms, indicating that the intermediate builds up at the ambient temperatures of the experiment. This represents the first direct detection of the intermediate with a native metal-dependent KDO8PS, and further demonstrates that time-resolved mass spectrometry is a useful tool in mechanistic studies of enzymatic reactions.

The molecule 3-deoxy-D-*manno*-octulosonate (KDO) is an eight-carbon sugar that forms an integral part of lipopolysaccharide (LPS) A in the cell membranes of gram-negative bacteria.[1,2] One of the enzymes involved in KDO production is 3-deoxy-D-*manno*-octulosonic 8-phosphate synthase (KDO8PS) which catalyzes the aldol condensation of arabinose-5-phosphate (A5P) and phosphoenolpyruvate (PEP) to form inorganic phosphate (Pi) and a precursor to KDO, 3-deoxy-D-*manno*-octulosonic 8-phosphate (KDO8P). A number of KDO8P synthases from different organisms have been identified and characterized.[2–6] These studies have revealed two distinct classes of KDO8PS: those that do not require a divalent metal ion for activity (metal-independent) and those that do (metal-dependent). The former includes KDO8PS from various *E. coli* strains, the latter includes KDO8PS from *Helicobacter pylori* (a human gastric pathogen) and the thermophilic organisms *Aquifex aeolicus* and *Aquifex pyrophilus*. Although the overall amino acid sequence conservation is low (30%) between different KDO8PS members, the crystal structures reveal that the folds and active site residues of the two classes are highly conserved. The exception is a cysteine in the metal- dependent enzymes that replaces an asparagine in the metal-independent enzymes.[1,7–9] The cysteine coordinates the metal, and it has been shown that the reciprocal mutations alter the metal-dependence of both classes of KDO8PS.[10] Structural and mechanistic studies have proposed that the metal facilitates

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proper orientation of substrates in the active site and/or facilitates communication between the four subunits that make up the homotetrameric KDO8PS.[1,2,8,11,12]

Detailed mechanistic information for the KDO8PS reaction has been obtained from the analysis of the pre-steady state reaction kinetics of metal-dependent and -independent KDO8PS using rapid chemical quenching and radiolabeled substrates. For all KDO8PS, product release is the rate-limiting step in the reaction, with the rate of chemistry ranging from 110 s^{-1} (*E. coli* at 25°C), to ~200 s^{-1} (*A. pyrophilus* at 60°C).[6,10,11] Early mechanistic work on *E. coli* KDO8PS proposed that the reaction proceeded through the formation of a noncovalently bound, acyclic hemiketal phosphate intermediate ([**I**], Fig. 1).[11] However, with no distinct spectroscopic signal, a short lifetime, and chemical lability, direct observation of this intermediate was expected to be difficult.

Since the development of electrospray ionization (ESI), mass spectral analysis of biological molecules has become routine, with increasing evidence suggesting that physiologically relevant structures are maintained in the gas phase. This is supported by numerous studies of noncovalent protein–protein and protein–small molecule complexes.[13,14] Coupled with its high sensitivity and accuracy, several groups recognized the potential for kinetic and mechanistic analysis of enzymatic reactions by mass spectrometry. This has included both direct and offline monitoring of reactions.[15–22] In particular, following reactions in the pre-steady state regime holds the greatest potential for identifying enzyme intermediates which are formed transiently in the active site.[23] To examine pre-steady-state kinetics directly, millisecond time resolution is often required, and a few different experimental setups for this type of analysis have been described.[24–27] Our laboratory developed a rapid-mixing attachment for online analysis of enzymatic reactions with a theoretical time resolution of 7 ms. The principles of the design are simple: syringes containing protein and substrate solutions are driven into a mixing tee by a syringe pump. The mixing tee is connected to a capillary (the reaction vessel) which terminates at the ESI source. A number of control experiments have shown that the kinetic constants derived from online rapid-mixing mass spectrometry vs. solution techniques are the same, indicating that quantitative rates may be obtained from online rapid-mixing mass spectrometry.[28,29] After careful optimization of gas pressures, temperatures, and capillary exit voltages, this mixing apparatus was used to detect the formation of a transient intermediate in the *E. coli* KDO8PS reaction under single turnover conditions, confirming the presence of a noncova-lent enzyme:intermediate complex with a mass consistent with the acyclic hemiketal phosphate. This provided the first direct evidence for this transient intermediate in the metal-independent class of KDO8PS.[29]

Although a recent crystal structure of a triple mutant of metal-dependent *A. aeolicus* KDO8PS revealed electron density in the active site consistent with the proposed reaction intermediate, direct detection of the intermediate under native conditions has not been achieved.[1,7–9,12] In this study we observed the proposed hemiketal phosphate intermediate in the metal-dependent KDO8PS reaction from *Aquifex pyrophilus* under single turnover conditions with time-resolved mass spectrometry. Distinct PEP, KDO8P, intermediate, and Pi species in noncovalent complexes with Cd^{2+}-bound KDO8PS are observed, supporting the broad applicability of time-resolved mass spectrometry as a tool for mechanistic studies of metalloenzymes.

EXPERIMENTAL

Enzyme expression and purification

A. pyrophilus KDO8PS was expressed and purified as previously described, with some exceptions.[10] Growth in minimal media (NaCl, potassium phosphate and ammonium chloride supplemented after autoclaving with $CaCl_2$, glucose, and $MgSO_4$) avoided the

introduction of Fe^{2+} into the active site which facilitates irreversible oxidation of the metal-coordinating active site cysteine (creating cysteinesulfonic acid). Growth in normal Luria Bertani medium results in the production of approximately equal amounts of unoxidized and oxidized protein with a mass difference of 48 a.m.u., which notably increases the complexity of acquired mass spectra. The cysteine sulfonic acid as the source of the +48 a.m.u. observed in the ESI-TOF mass spectra was validated by liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis (data not shown) and was also observed in the crystal structure of the homologous *A. aeolicus* KDO8PS.[8] Growth in minimal media resulted in the production of predominantly unoxidized KDO8PS. To avoid further oxidation during the purification process, all buffers for purification were degassed with N_2, were supplemented with 100 μM Cd^{2+}, and purification was performed over the course of 1 day. Aliquots of purified *A. pyrophilus* KDO8PS (300–500 μM) were flash frozen in liquid N_2.

Preparation of enzyme and substrates for mass spectrometry

Aliquots of *A. pyrophilus* KDO8PS were mixed with equimolar amounts of cadmium acetate (Sigma) and PEP (Sigma) that had been desalted via Q Sepharose ion-exchange chromatography into 20 mM ammonium acetate, pH 7.1, and were allowed to incubate on ice for 30 min. To exchange the protein into a volatile buffer for mass spectrometry, these mixtures were then successively applied to two Biorad P6 spin columns pre-equilibrated with 50 mM ammonium acetate, pH 7.2 (Biorad). The concentration of the resulting enzyme solution was measured via UV spectrometry using the calculated extinction coefficient of 20460 M^{-1} cm^{-1} at 280 nm. The enzyme, prebound with Cd^{2+} and PEP, was diluted to 80 μM with 25 mM ammonium acetate, pH 7.2. A5P (Sigma) was desalted using an Alltech exchange column as previously described and diluted to 70 μM using 25 μM ammonium bicarbonate, pH 8.[29] For each experiment, a blank (containing no A5P), and a reaction mixture containing premixed enzyme:Cd^{2+}:PEP, was analyzed to assess the ratio of bound species at equilibrium.

Single turnover experiments using rapid chemical quench and radioactive detection of product

A. pyrophilus KDO8PS was prepared as described for mass spectrometry experiments (80 μM) except that 60 μM of radiolabeled substrate [1-^{14}C]PEP (6.5 Ci/mol), prepared as previously described, was added after passage through a Biorad P6 spin column.[10] This mixture was combined with the second substrate, A5P (80 μM in 25 mM ammonium bicarbonate), in a Kintek Rapid-Chemical Quench for different time periods at 25°C, and the reactions were quenched with 0.6 M KOH. The resulting quenched samples were run on a MonoQ anion-exchange HPLC column (Amersham) equilibrated with 20 mM triethyl ammonium bicarbonate (TEAB), pH 7.8. The substrate (PEP) and product (KDO8P) were eluted with a gradient of 20 mM to 1 M TEAB. The high-performance liquid chromatography (HPLC) system was interfaced with an inline radioactivity flow detector (Perkin-Elmer) to allow real-time quantitation of radiolabeled substrate and products. The fraction of product formed was determined by cpm (KDO8P)/cpm (KDO8P + PEP).

Mass spectrometry data collection and processing

All mass spectrometry experiments were performed on an Ettan ESI-TOF (Amersham/Pharmacia). The rapid-mixing ESI source has been previously described and was custom-made by Perkin Elmer (formerly Analytica of Branford).[29] The mixing capillary was composed of fused silica with an inner diameter of 30 microns. For kinetic experiments, the following parameters were used for data acquisition: drying gas 180°C (at 10–13 L/min.), nebulizing gas (4–4.6 bar), exit voltage of 140 V and skimmer 20 V (observation of the intermediate particularly depends on these last two parameters). Spectra collected at

different mixing times were averaged, and a baseline correction and smoothing functions were applied in the software (Aviator, Analytica of Branford.)

RESULTS AND DISCUSSION

As a first step in designing experimental conditions to look for the labile acyclic hemiketal phosphate intermediate using time-resolved ESI-MS, we examined the reaction time course under single enzyme turnover conditions using rapid chemical quench methodology. In this assay, the formation of radiolabeled product was monitored at different time points via HPLC and radioactive detection. Single turnover conditions were achieved by incubating the enzyme with a substoichiometric amount of [1-^{14}C]PEP and equimolar unlabeled A5P. In this type of experiment, adding enzyme in excess of radiolabeled substrate enhances the possibility of detecting intermediate species that may be formed in low abundance at the enzyme active site.[30] In this case, the anticipated acyclic hemiketal phosphate would not be predicted to be stable under the chemical quench conditions employed; however, the time frame for examining chemical catalysis using mass spectrometry could be established.

Rapid chemical quench

Previous rapid chemical quench experiments determining the reaction kinetics of thermophilic *A. pyrophilius* KDO8PS at 60°C showed that the rate of catalysis was very rapid (~200 s^{-1}).[10] The reaction kinetics at 25°C were examined with the hypothesis that a slower reaction time might facilitate buildup of the intermediate. In adapting these conditions to conduct time-resolved mass spectrometry experiments, an added benefit for the longer mixing times would be lower flow rates in the ESI source which generally results in spectra with narrower linewidths and an increased signal-to-noise ratio.

A single turnover reaction was performed at 25°C with rapid chemical quench to establish the reaction kinetics for conversion of substrates, A5P and PEP, to products, KDO8P and Pi. The reaction time course is shown in Fig. 2. A fit of the data to a single exponential equation provided a reaction rate of 24.3 s^{-1}. Accordingly, for this catalytic rate, the corresponding half-life ($t_{1/2}$) is 25 ms. This rapid chemical quench experiment allowed us to approximate conditions that would be employed for the time-resolved mass spectrometry experiment designed to look for an enzyme intermediate.

Time-resolved rapid-mixing mass spectrometry

Using the time frame established by rapid chemical quench experiments, we examined the catalysis of *A. pyrophilus* KDO8PS at 25°C using time-resolved mass spectrometry at reaction times ranging from 40 ms (flow rate of 50 μL/min) to 630 ms (flow rate of 2.5 μL/min.) A series of averaged mass spectra collected at different time points are shown in Fig. 3. The bottom spectrum (labeled blank in Fig. 3) represents the control data for KDO8PS complexed with Cd^{2+} and PEP and in the absence of A5P. In this experiment, noncovalent enzyme-ligand complexes are observed as peaks corresponding to the apo protein (KDO8PS), KDO8PS:Cd^{2+}, and KDO8PS:Cd^{2+}:PEP (+167) (See Table 1). A small shoulder on these peaks is the result of residual oxidation of the active site cysteine (+48 a.m.u.). The cadmium-bound enzyme also has a peak corresponding to a complex with Pi (within experimental error). This may be a contaminant in the PEP preparation. Additionally, it is known that Pi (that is produced in the reaction) is a competitive inhibitor of PEP and A5P.[12] The metal of choice (Cd^{2+}) was selected because this metal:enzyme complex results in optimal activity of the enzyme. In preparation of the KDO8PS:Cd^{2+}:PEP noncova-lent complex, we wanted to maximize the amount of PEP bound to the enzyme. It was found that pre-binding the enzyme with PEP before desalting yielded a higher degree of complexation than adding PEP to the already desalted, Cd^{2+}-bound enzyme.

As seen in Fig. 3, there is a noticeable difference in the relative intensities of the apo KDO8PS (represented by R) and KDO8PS:Cd^{2+} complex (represented by ●) at different time periods. This is observed particularly in the blank control relative to the other trials in which catalysis is occurring. The fraction of Cd^{2+}-bound enzyme was very sensitive to buffer conditions. Using only ammonium acetate buffer, nearly all the observed KDO8PS was bound to Cd^{2+}, but the spectra had poor resolution. When the enzyme in ammonium acetate buffer was mixed with ammonium bicarbonate buffer, the resolution of the spectra greatly improved, but a lower percentage of Cd^{2+}-bound enzyme was observed (data not shown). In the crystal structure of *A. aeolicus* KDO8PS, both PEP and A5P form extensive electrostatic interactions with residues or water molecules in the vicinity of the metal ion. Presumably, the addition of both substrates might stabilize the metal-bound form of the enzyme, resulting in a relative increase in the intensity of this species versus the free enzyme.[8] In the 50–300 ms time range there is roughly a 12% variation in the relative intensities of Cd^{2+}-bound and apo enzyme, with a slight increase in the fraction of Cd^{2+}-bound enzyme at longer reaction times. This is most likely due to the "dilution" of the pure KDO8PS:Cd^{2+} complex by the additional complexes that form (substrate, product, and intermediate) simultaneously from the KDO8PS:Cd^{2+} complex.

Upon addition of stoichiometric/sub-stoichiometric concentrations of A5P and reaction times ranging from 50– 630 ms, several new species consistent with product formation are observed in addition to those in the control experiment (with mass differences relative to the Cd^{2+}-bound enzyme indicated). These include KDO8PS:Cd^{2+}:Pi(+102), KDO8PS:Cd^{2+}:KDO8P (+318), and a mass (+416) corresponding to KDO8PS:Cd^{2+}:[**I**] ([**I**] – hemiketal intermediate shown in Fig. 1). Some of the complex peaks observed are broad and of low intensity, contributing to less well-defined *m/z* values and small mass errors (Table 1).

As shown in Fig. 3, at progressively longer times, the KDO8PS:Cd^{2+}:PEP complex (represented by ■ in Fig. 3) decreases while the product complexes (KDO8PS:Cd^{2+}:KDO8P) and (KDO8PS:Cd^{2+}:Pi), (represented by ○ and Δ, respectively, in Fig. 3) increase. Also in these time courses, a peak is observed that is consistent with KDO8PS:Cd^{2+}:[**I**] (represented by * in Fig. 3) in accord with the disappearance of PEP substrate and formation of products, KDO8P and Pi, suggesting that the intermediate is formed in a kinetically competent manner. The mass for the intermediate could also correspond to the product complex (KDO8P + Pi); however, attempts to incubate the protein with KDO8P and Pi did not lead to formation of a viable complex (data not shown.) This is consistent with kinetic and structural data from *A. aeolicus* KDO8PS indicating that as soon as the products are formed, the binding site for Pi is essentially obliterated due to weak binding affinity, with a correspondingly large k_{off} rate.[12] Additionally, longer (minutes) preincubation of KDO8PS:PEP:Cd^{2+} and A5P yielded only KDO8PS:Cd^{2+}:K-:KDO8P complexes, with no other observable species corresponding either to KDO8P + Pi, or intermediate. The intermediate also has a mass consistent with both substrates plus a water molecule, but given the rate of chemistry and that the product is formed at the earliest timepoints, this complex is unlikely to be observed.

There is a variation in the signal intensity at different timepoints (due to desolvation efficiency being greater at lower flow rates) that makes quantitation of the observed signals somewhat complex. However, if the signal intensity of the KDO8PS:Cd^{2+}:KDO8P complex at each time point in Fig. 3 is divided by the sum of the intensities of the KDO8PS:Cd^{2+}:KDO8P and KDO8PS:Cd^{2+}:PEP signals, the data can be fit to a single exponential with a rate of chemistry of 18.2 ± 0.6 s^{-1}, in general agreement with the rates observed by chemical quench with radioactive detection.

CONCLUSIONS

We report here the direct observation of a transient, labile hemiketal enzyme intermediate in the reaction of metal-dependent *A. pyrophilus* KDO8PS using time-resolved mass spectrometry at a physiological pH with wild-type enzyme. The observation of multiple noncovalent complexes and their time-resolved profiles illustrates the utility of this approach in probing enzymatic reactions. Moreover, this study shows that time-resolved mass spectrometry is a powerful methodology for detecting intermediates, especially if these species are formed transiently and are chemically labile.

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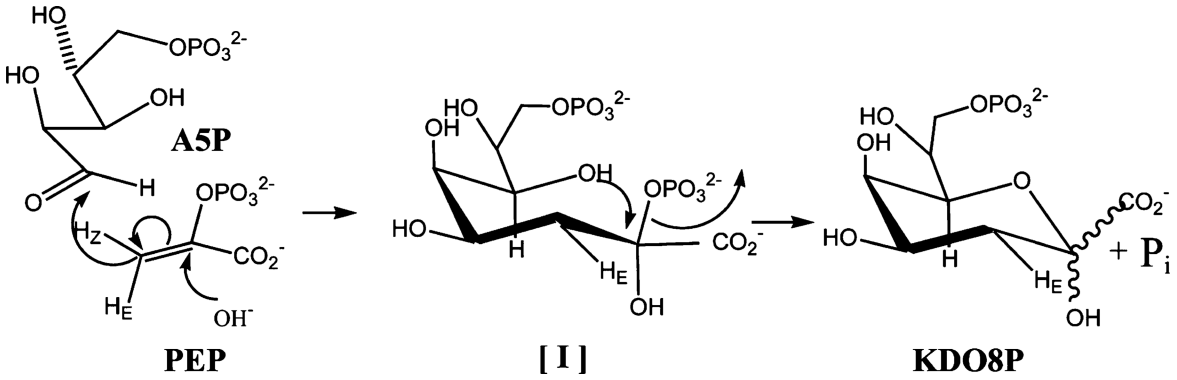

Figure 1.
Reaction of A5P with PEP proceeds through the formation of an acyclic hemiketal intermediate [**I**].[11]

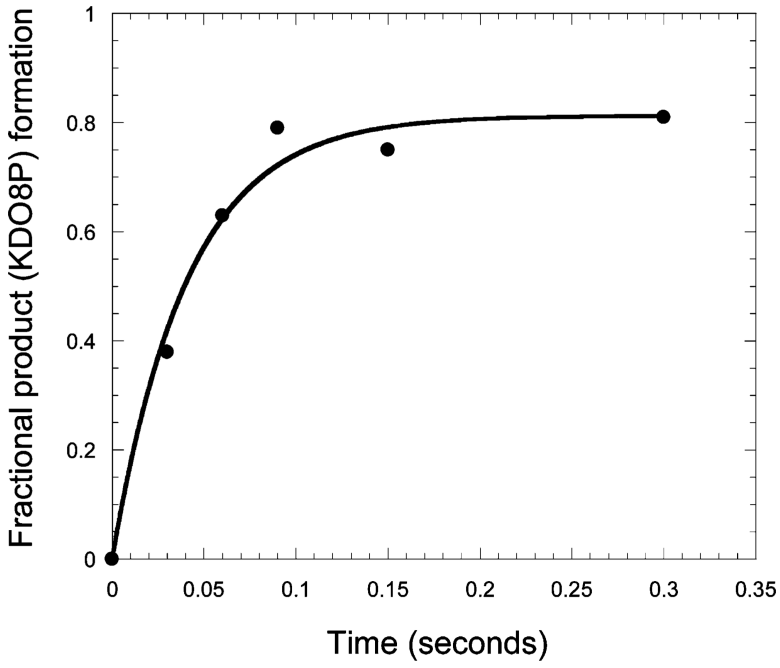

Figure 2.

Single turnover reaction of *A. pyrophilus* KDO8PS usingrapidchemicalquenchcoupledwithradioactivedetection, under theconditionsusedfor time-resolvedmassspectrometry. The data were fit to a single exponential corresponding to a rate of chemistry of 24.3 ± 3.5 s^{-1}, with an amplitude of 0.8, representing the fractional concentration of active sites.

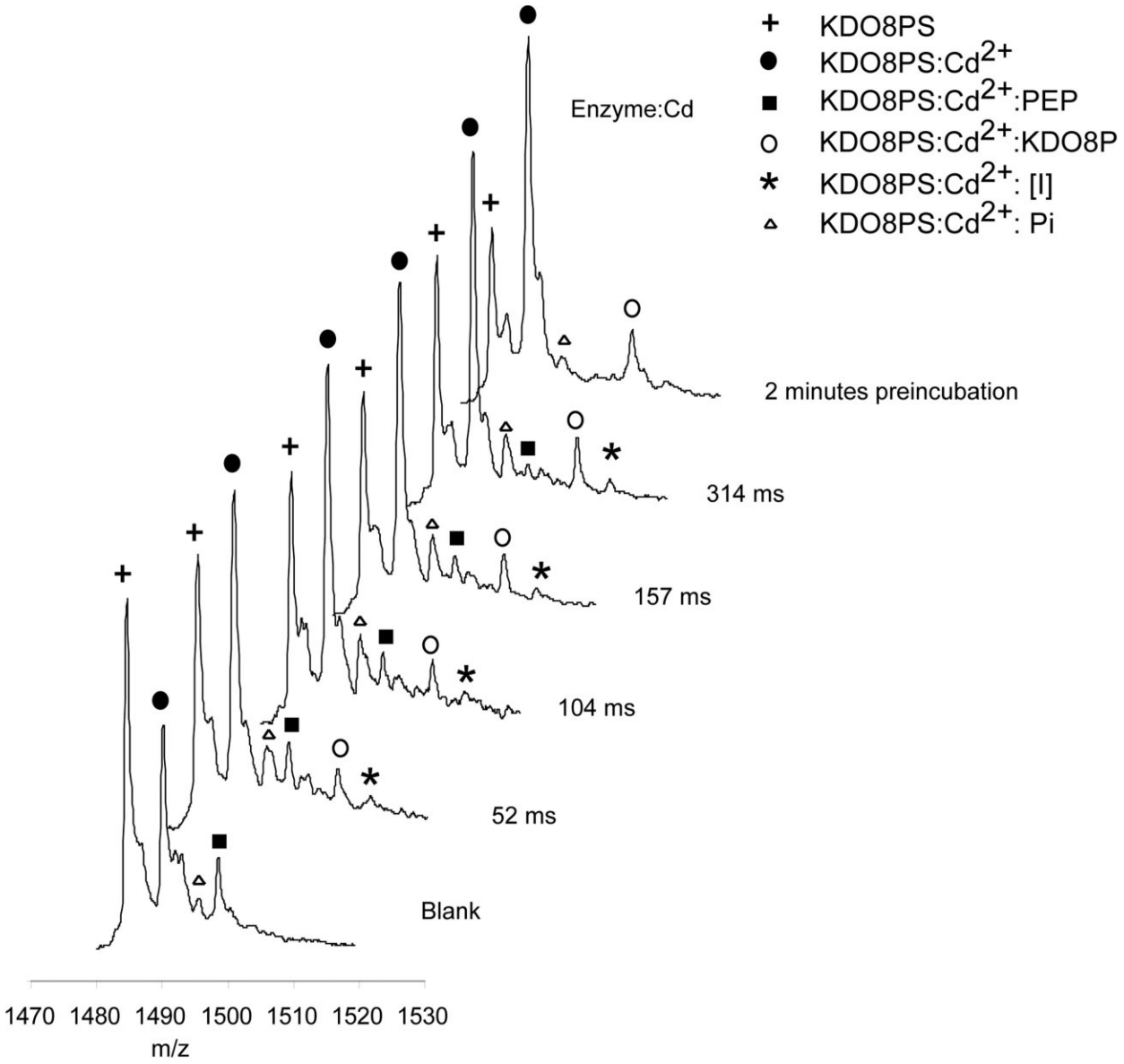

Figure 3.
Time-resolved mass spectra of the reaction of metal-dependent *A. pyrophilus* KDO8PS monitored at charge state +20. The reaction times and complexes observed are indicated.

Table 1

Representative complex peaks at charge state +20. Expected versus calculated molecular weight differences are indicated. The expected PEP Pi, KDO8P and [I] mass differences (Δm) are relative to the Cd^{2+}-bound enzyme

	m/z (*z* +20)	Molecular weight	Δm	Observed Δm
KDO8PS	1485.161	29678.8		
KDO8PS:Cd^{2+}	1490.406	29787.5	110	108 ± 2
KDO8PS:Cd^{2+}:PEP	1498.878	29954.2	167	167 ± 4
KDO8PS:Cd^{2+}:Pi	1495.183	29890.4	96	102 ± 7
KDO8PS:Cd^{2+}:KDO8P	1506.394	30105.3	315	318 ± 1
KDO8PS:Cd^{2+}:[I]	1510.906	30202.6	411	416 ± 1