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Induced fit activity-based sensing: a mechanistic study of pyrophosphate detection with a “flexible” Fe-salen complex†

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Activity-based sensing of biological targets is attracting increasing attention. In this work, we report detailed UV-Vis and fluorescence mechanistic studies on an Fe-salen based probe, [Fe^{III}(salenMeCl₂(SO₃)₂)OH₂]⁻ for pyrophosphate (PPI) detection. In the presence of PPI as an analyte, the probe disassembles into its molecular subunits and releases a fluorescent signal. Our studies illustrate that the aqua form of the complex (**1-OH₂**) is the active species and that upon substitution of Fe-coordinated H₂O and an initial end-on coordination of HP₂O₇³⁻, the “trapped” pyrophosphate species switches from a monodentate to a bidentate coordination mode (*i.e.* linkage isomerism) *via* a probable equilibrium process. The elusive intermediate is further stabilized by a hydrogen bonding interaction that activates the probe for the subsequent final irreversible rate-limiting step, and allows selective discrimination between the other pyrophosphate (H₂P₂O₇²⁻ and P₂O₇⁴⁻) species in favour of the HP₂O₇³⁻. The flexible mode of molecular recognition and binding of HP₂O₇³⁻ by the tetradentate probe **1-OH₂** is unexpected and most effective at physiological pH, and has precedence in enzymatic catalysis (*i.e.* induced fit principle). These binding properties explain the previously observed outstanding selectivity of **1-OH₂** for pyrophosphate over other (poly)oxophosphates and potentially competing analytes.

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Introduction

Activity-based sensing (ABS) attracts increasing attention for elucidating the roles and importance of chemical species in biological systems.^{1,2}

In this context, enormous progress has been observed in the development and application of chemosensors for detecting small reactive molecules of the carbon-,^{3,4} oxygen-,^{5,6} nitrogen-⁵⁻⁷ and sulfur families.^{6,8,9} In contrast to these developments, chemical methods for sensing phosphorus containing compounds in biological environments are scarce.¹⁰⁻¹³ Phosphates are ubiquitous in biological systems and play pivotal structural and functional roles ranging from its occurrence in the skeleton to signal transduction and energy storage processes.^{14,15} These biologically important anions are present in multiple facets and phosphates are encountered in different

protonated forms of inorganic mono- and polyanions or as covalently bound building blocks of natural products such as pyrophosphate (PPI),¹⁶ adenosine triphosphate (ATP) or vitamin B₁₂.^{17,18} For example, H₄P₂O₇ is a four-proton donor with pK_a values of 0.85, 1.96, 6.60 and 9.41¹⁹ and hence, at physiological pH of 7.40, PPI is encountered as a mixture of HP₂O₇³⁻ (86%) and H₂P₂O₇²⁻ (14%).^{11,20}

The Zelder group and others are interested in the development of activity-based probes to detect and study the roles of phosphates in biological systems, in the environment and in foodstuff.^{11,21-23} For this purpose, fluorescent probes are advantageous, but selective sensing of the target analyte in the presence of a pool of related bioavailable, competing phosphates is highly challenging.²⁴⁻²⁷ Genetically encoded and aptamer based systems offer interesting opportunities,²⁸⁻³⁰ whereas cost-effective small-molecular chemical systems are still rare. Important examples of the latter include a boronic acid functionalized rhodamine derivative for detecting mitochondrial ATP as well as Zn-terpyridine system for sensing cellular PPI.^{27,31} Two of us introduced Fe-salen complexes for selectively detecting PPI in cancer cells and in foodstuff.^{11,21,23} The Fe^{III} complex **1-OH₂** (Fig. 1A) exhibits a square pyramidal coordination geometry and is composed of four (i-iv) subunits:²³ a central Fe^{III}-ion (i), a 1,2-propanediamine backbone (6) (ii), two 3-chloro-5-sulfosalicylaldehyde moieties (4) (iii)

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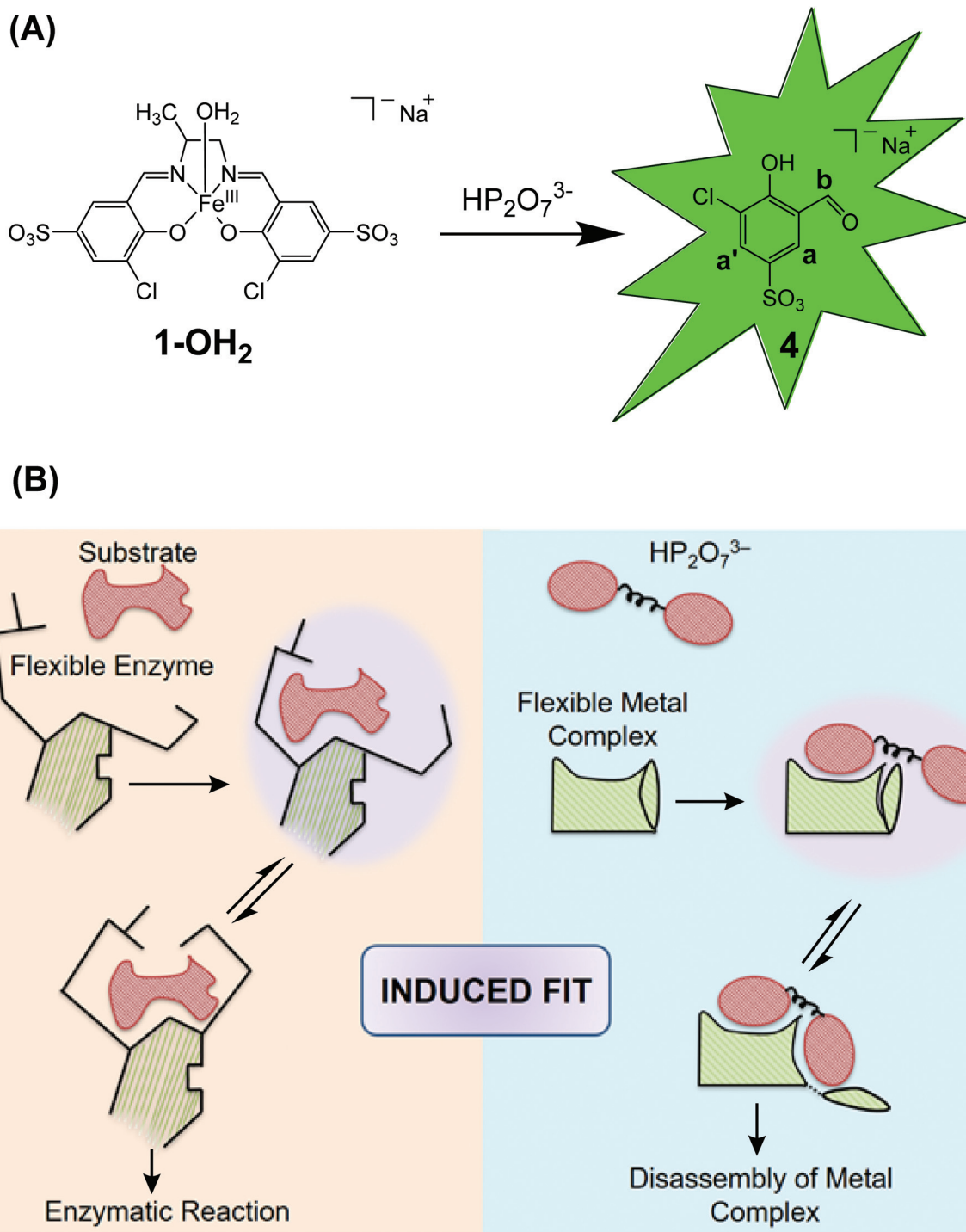


Fig. 1 A: Liberation of the signaling unit 4 upon disassembly of the Fe-complex **1-OH₂** with $\text{HP}_2\text{O}_7^{3-}$. B: Schematic comparison of the induced fit mechanism in enzymatic catalysis³² with the analyte-triggered disassembly of a “flexible” metal complex as described in this study for the reaction between **1-OH₂** and $\text{HP}_2\text{O}_7^{3-}$.

and an apical water ligand (iv; Fig. 1A).²³ Upon interaction with PPI, the metal complex disassembles and unleashes the fluorescent signaling unit 4 (Fig. 1A).²³ Importantly, discrimination between PPI and other anions including related phosphates such as inorganic phosphate, ATP and ADP was demonstrated.²³ Mechanistic details on the nature of the active

species, the molecular recognition and the binding event as well as the irreversible disassembly process have however not been studied so far.

Thus, herein we present a detailed kinetic-mechanistic study on the structurally “flexible” probe **1-OH₂** for the fluorometric detection of pyrophosphate (Fig. 1). In particular, we



unravel an unprecedented reorganization of the Fe^{III}-active site of **1-OH₂** upon binding of the analyte (*i.e.* HP₂O₇³⁻; Fig. 1B right). This PPI-induced structural change of the iron binding site of **1-OH₂** is a pre-requisite for triggering the subsequent decomposition of the probe and liberation of the fluorescent signaling unit **4**. The initial recognition and binding process of PPI by **1-OH₂** is reminiscent to the induced fit binding of substrates by “flexible” enzymes such as phosphoglucomutase described with Koshland’s induced fit theory (Fig. 1B left).^{32,33}

Experimental section

Materials

Complex **1-OH₂** was synthesized as its sodium salt as reported earlier.²³ Sodium salts of PPI and F⁻ were used in the kinetic studies. Unless otherwise stated, all chemicals were of reagent grade and purchased from Sigma-Aldrich-Merck, Fluka, Apollo Scientific or Alfa Aesar. Solvents for reactions were of p.a. grade. Stock solutions for kinetic experiments were prepared in Milli-Q H₂O.

Instrumentation

pH: Metrohm 827 pH meter equipped with pH sensitive electrode was employed. UV-Vis spectra: Cary Series Spectrophotometer (Agilent technologies); λ_{max} in nm. UV spectra were recorded between 230 and 800 nm at 1.2 nm resolution and 20 points per s. Fluorescence spectra: Luminescence spectrometer, PerkinElmer, LS 50B. Unless stated, the slit-widths for the fluorescence experiments were kept at 10 nm (excitation) and 10 nm (emission) and the excitation wavelength was set at 385 nm. Unless stated, a UV Fused Silica Metallic Neutral Density Filter (FSQ-ND05) with optical density of 0.5 (from Newport) was used for fluorescence studies. ¹H-NMR spectra in D₂O; Bruker AV-401 (400 MHz) or Bruker AV2-500 (500 MHz); *d* in ppm rel. to TMS (*d* 0.00), *J* in Hz.

Kinetic studies and data treatment

All UV-Vis and fluorescence kinetic experiments were carried out in a quartz cuvette (3.5 mL, 10 mm) without protection from air under pseudo first-order reaction conditions. Unless otherwise stated, all kinetic experiments were carried out with complex **1-OH₂** (16 μ M) at pH 7.35 ([Tris buffer] = 10 mM) at five different temperatures (12, 18, 25, 32 and 38 °C) and in presence of seven different concentrations of PPI (0.16, 0.24, 0.32, 0.48, 0.56, 0.64 and 0.80 mM). pH studies were performed at thirteen different pH values: 4.50, 5.00, 5.36, 5.71, 6.02, 6.33, 6.70, 7.00, 7.35, 7.67, 8.12, 8.42 and 9.40 ([Britton Robinson buffer] = 10 mM). Absorption and emission traces were collected over time until completion of reaction indicated by saturation. Origin 2020 and Micromath Scientist³⁴ were used for fitting the kinetic traces and other specific functions. The observed pseudo first-order rate constants were obtained from least-squares (L.S.) fits of absorbance at 385 nm and emission at 500 nm ($\lambda_{\text{ex}} = 385$ nm) vs. time traces to an appro-

priate first-order exponential,³⁵ as described previously and illustrated in Fig. 3 and ESI†^{36–41} In Fig. 3–6, the solid lines indicate the L.S. fits/simulations of the respective experimental data (shown as individual points) to appropriate functions (details shown in the ESI†).

Computational details

Density functional theory (DFT) calculations were carried out using the Gaussian 09 program package.⁴² The hybrid functional B3LYP⁴³ was chosen in association with the standard 6-31G(d) basis set⁴⁴ and the conductive polarizable continuum model (CPCM)^{45,46} for the solvent effects (water). The optimized ground state structures were obtained by unrestricted calculations and confirmed to be potential energy minima by vibrational frequency calculations at the same level of theory (no negative frequency).

Results and discussion

PPI-triggered disassembly of complex **1-OH₂**

The PPI-triggered disassembly of **1-OH₂** into the signaling unit 3-chloro-5-sulfosalicylaldehyde (**4**), 1,2-propanediamine (**6**) and Fe(PPI)_{*n*} (**5**) (Scheme 1) was established previously based on the following observations.²³ Firstly, upon addition of PPI (10 equiv.; 0.16 mM) to an aq. soln. of **1-OH₂** (16 μ M), the formation of a new absorption band at 385 nm was observed in the UV-Vis spectrum and a simultaneous 3.5-fold enhanced emission at 500 nm was observed with fluorescence spectroscopy (see also Fig. 3).²³ These characteristic spectroscopic changes were unambiguously attributed to the liberation of **4** from complex **1-OH₂**.²³ Moreover, these observations were supported by mass spectrometric and ¹H NMR studies.²³ In the latter experiments, formation of **4** from **1-OH₂** (2 mM) was observed within 30 min at pD 7.35 ([Tris buffer] = 10 mM) after the addition of PPI (10 equiv.; 20 mM). Remarkably, we did not obtain any evidence for the intermediate formation of the metal-free salen ligand from disassembly experiments in deuterated H₂O (Fig. 2).

Kinetic studies of the disassembly mechanism

Fig. 3A reports UV-Vis spectral changes for the reaction between complex **1-OH₂** (16 μ M) and PPI (30 equiv.; 0.48 mM) at pH 7.35 ([Tris buffer] = 10 mM) and 25 °C as a function of time. Changes of absorbance at 385 nm indicate the release of the signaling unit **4** and depict a first-order behavior with a rate constant of $k_{\text{obs(Abs)}} = (1.025 \pm 0.008) \times 10^{-3} \text{ s}^{-1}$ and a half-life of *ca.* 600 s (approx. 10 min) (Fig. 3 inset). Also shown in Fig. 3 is the concurrent fluorescence changes at 500 nm ($\lambda_{\text{ex}} = 385$ nm) with time (at 28 °C), which is clearly very similar to that observed from the absorbance measurements with $k_{\text{obs(Flu)}} = (1.30 \pm 0.03) \times 10^{-3}$ (single exponential) and $k_{\text{obs(Flu)}} = (1.280 \pm 0.006) \times 10^{-3}$ (two exponential) s⁻¹, respectively, taking into account the slight temperature increase.



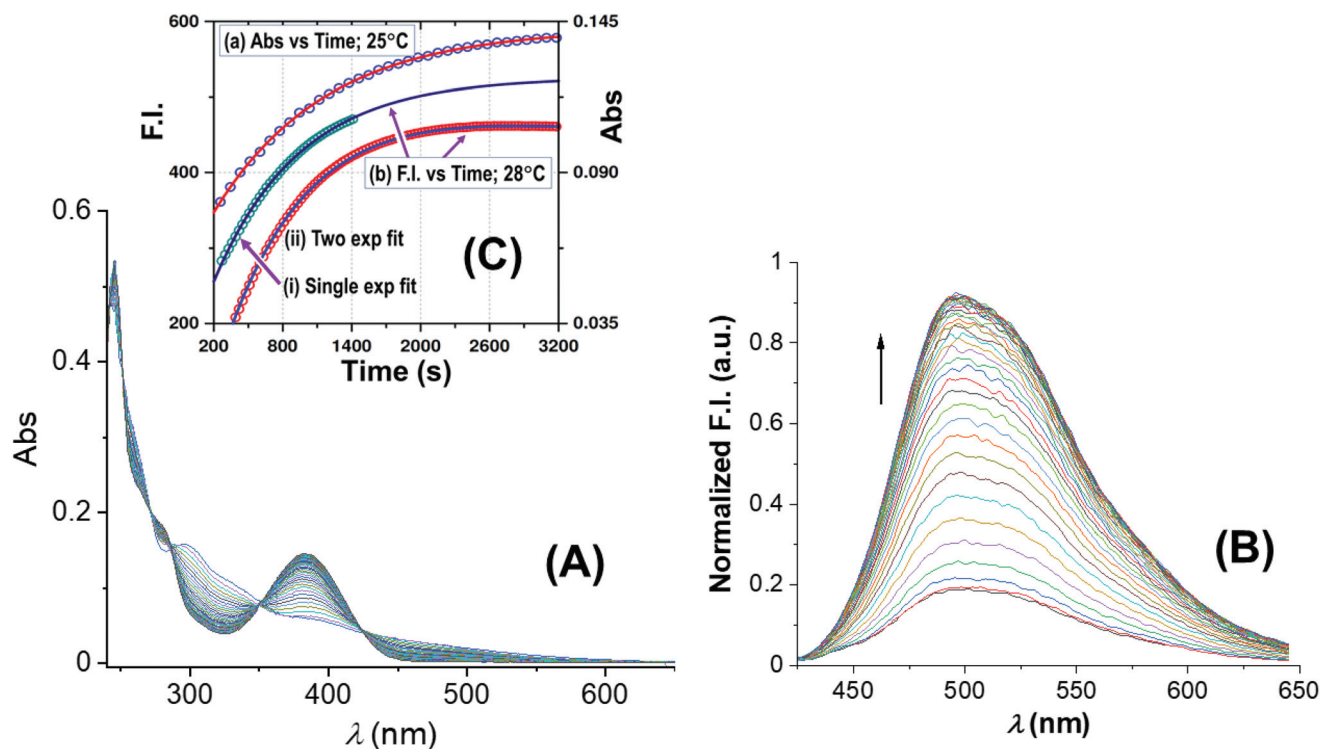


Fig. 3 Spectral changes of the reaction between **1-OH₂** (16 μM) and PPI (30 equiv.; 0.48 mM) with time at pH 7.35 ([Tris buffer] = 10 mM) at 25 °C: (A) absorbance vs. time scans; (B) fluorescence vs. time scans at 28 °C; inset (C): associated time traces of changes in absorbance at 385 nm and fluorescence emission at 500 nm ($\lambda_{\text{ex}} = 385$ nm) illustrating similar kinetics, L.S. fitted to a typical first-order exponential (see text), yielding values for (a) $k_{\text{obs(Abs)}} = (1.025 \pm 0.008) \times 10^{-3} \text{ s}^{-1}$, and (b) for $k_{\text{obs(Flu)}} = (1.30 \pm 0.03) \times 10^{-3}$ (single exponential) and $k_{\text{obs(Flu)}} = (1.280 \pm 0.006) \times 10^{-3}$ (two exponential) s^{-1} , respectively (text; ESI Fig. S5†). Note that for clarity the y-axis of the single exponential F.I. trace indicated by (i) has been shifted by ca. +100 F.I. units to ensure no overlap with trace (ii) in the illustration.

nucleophilic for substituting the aqua ligand in complex **1-OH₂** ($\text{p}K_{\text{a}} = 6.90$)¹⁹ as indicated by the rapid decrease of the reaction rates at more acidic pH values (<pH 6.00). This underlines the novel ability of **1-OH₂** to selectively discriminate between the $\text{H}_2\text{P}_2\text{O}_7^{2-}/\text{P}_2\text{O}_7^{4-}$ anions (both present in significant amounts in the pH range studied) in favor of $\text{HP}_2\text{O}_7^{3-}$. Moreover, it agrees with the fact that at more basic pH values, even the presence of the highly charged $\text{P}_2\text{O}_7^{4-}$ species ($\text{p}K_{\text{a4}} = 9.41$)¹⁹ does not trigger the disassembly reaction of **1-OH₂**, which might in turn suggests that hydrogen bonding in the ring closing step is important (see further below). It should be noted that there is an indication of a slow parallel reaction (data points at ca. pH 8.00–9.00), although it clearly does not significantly affect neither the specificity nor the selectivity of **1-OH₂**.

Having determined the aqua form of Fe-complex (**1-OH₂**) and $\text{HP}_2\text{O}_7^{3-}$ as the active species in the disassembly reaction, we studied the influence of increasing concentration of $[\text{PPI}]_{\text{tot}}$ on the reaction rate at the pH where the capturing of the $\text{HP}_2\text{O}_7^{3-}$ is the most effective. Variations of $[\text{PPI}]_{\text{tot}}$ at pH 7.35 and 25 °C (Fig. 5 blue graph) provide evidence of faster, preceding reactions, *i.e.*, saturation kinetics with a rate-limiting dependence on $[\text{PPI}]_{\text{tot}}$. The saturation kinetics provides excellent support for concluding preceding reactions, as postulated in Fig. 1 and Scheme 1.^{49,50} This behavior is thus supporting a rapid overall pre-equilibrium reaction, denoted by K_{eq} and

within this report indicated to be the net of initial coordination (K_1) followed by a linkage isomerism (K_2) succeeded by a final rate limiting first-order formation of products **4** and **5** (Scheme 1), denoted by k_{PPI} . Based on these observations and the detailed study of the following systematic kinetic experiments, we summarize the mechanism of the PPI-triggered disassembly of Fe-salen complex **1-OH₂** into its molecular building blocks **4**, **5** and **6** in Scheme 1.

The total rate law may be derived from Scheme 1, yielding the complete expression of the pseudo first-order rate constant in eqn (1), which describes all the observations made in this study related to the total process.

$$k_{\text{obs}} = k_{\text{PPI}}K_{\text{eq}}[\text{HP}_2\text{O}_7^{3-}]/\{1 + (K_{\text{a}}/[\text{H}^+]) + (K_{\text{eq}}[\text{HP}_2\text{O}_7^{3-}])\} \quad (1)$$

In eqn (1), the concentration of the tri-anionic species $\text{HP}_2\text{O}_7^{3-}$ is given by eqn (2).

$$[\text{HP}_2\text{O}_7^{3-}] = \{[\text{PPI}]_{\text{tot}}(K_{\text{a3}}/[\text{H}^+])\}/\{1 + (K_{\text{a3}}/[\text{H}^+]) + (K_{\text{a3}}K_{\text{a4}}/[\text{H}^+]^2)\} \quad (2)$$

In particular, the net total reactivity and specificity of **1-OH₂** towards the tri-anionic $\text{HP}_2\text{O}_7^{3-}$ species is well addressed in eqn (1), when $[\text{PPI}] \gg [\mathbf{1}]_{\text{tot}}$, and clearly describes



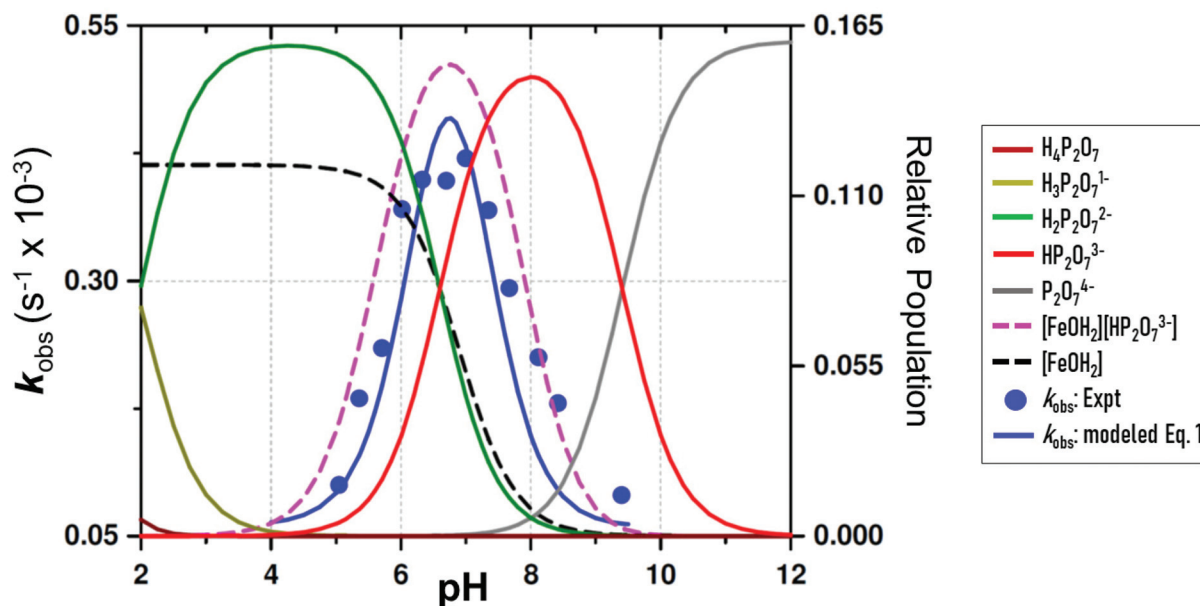


Fig. 4 pH dependence of the observed pseudo first-order rate constant (k_{obs}) (blue points) for the reaction between 1-OH₂ (16 μM) and PPI (10 equiv.; 0.16 mM) at 25 $^{\circ}\text{C}$. The line (blue curve) shows a modeled fit from all data at 25 $^{\circ}\text{C}$, including those obtained from the fluorescence traces at 28 $^{\circ}\text{C}$ to the overall total rate law expression, eqn (1). This was obtained using $k_{\text{PPI}} = 3.1 \times 10^{-3} \text{ s}^{-1}$; and $K_{\text{eq}} = 2700 \text{ M}^{-1}$, as well as a slow reaction concurrent to k_{PPI} , as intercept, $k_{22} = 6.0 \times 10^{-5} \text{ s}^{-1}$, to model the best visual fit [restrained to within the large relative standard deviations as expected: (i) due to the complexity of the system and model utilised, and (ii) having two dependent variables simultaneously] for the parameters k_{PPI} and K_{eq} . Also shown are relative speciation curves of PPI vs. pH, correlated to the pH dependency of k_{obs} , as well as the pH speciation of 1-OH₂ (black). These are all compared to the product of the populations of 1-OH₂ and HP₂O₇³⁻ (pink).

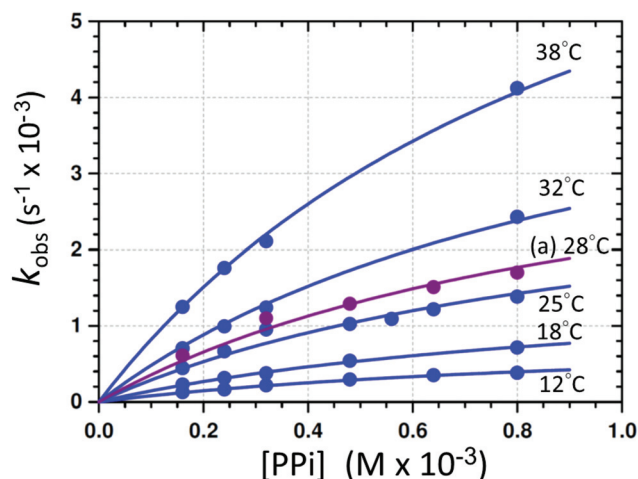


Fig. 5 Individual L.S. fits of k_{obs} vs. $[\text{PPI}]_{\text{tot}}$ (eqn (1)) at different temperatures and pH 7.35 ([Tris buffer] = 10 mM). (a) The purple line indicates the profile obtained from the F.I. vs. time traces at 28 $^{\circ}\text{C}$. A global fit was additionally performed to determine the activation parameters (sections S1.1, S4, and Fig. S6[†]).

(a) The total acid–base behavior of both the PPI and the 1-OH₂ species (Fig. 4);

(b) The plateau reached at high $[\text{PPI}]_{\text{tot}}$ (Fig. 5);⁵¹

(c) The simultaneous net changes in absorbance and fluorescence induced (Fig. 3).

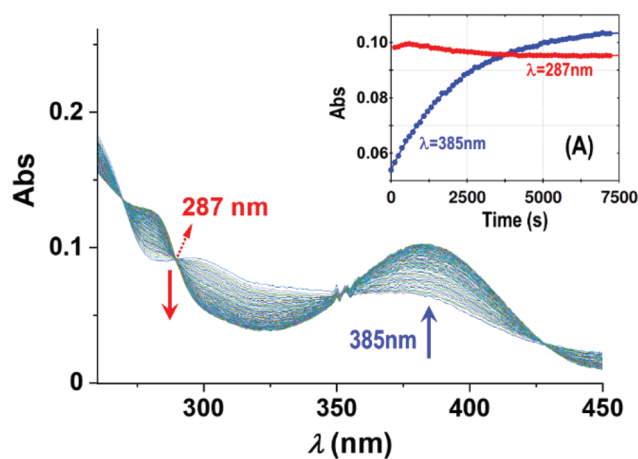
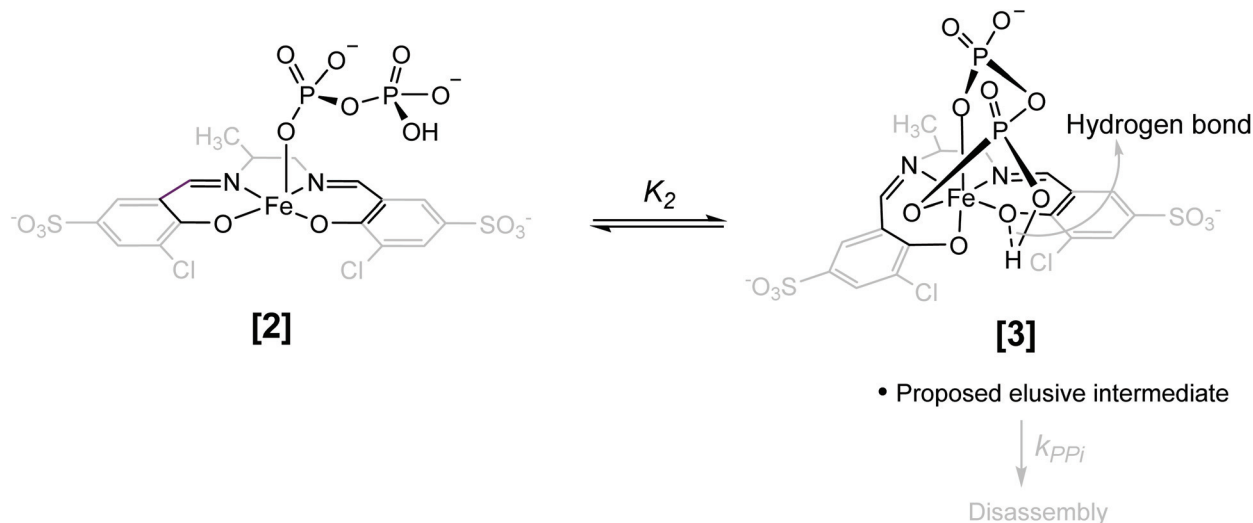


Fig. 6 Absorbance changes of 1-OH₂ (16 μM) around the isosbestic point (287 nm) with time in the presence of PPI (10 equiv.; 0.16 mM) at pH 7.35 ([Tris buffer] = 10 mM) and 12 $^{\circ}\text{C}$ illustrating the presence of earlier reactions. Inset (A): L.S. fits of Abs vs. time at 287 nm (red) and 385 nm (blue), respectively, showing the small absorbance changes of the first reactions (induction period) and underlining the small absorbance changes therewith, not influencing the individual fits and kinetic data as illustrated in the k_{obs} vs. $[\text{PPI}]_{\text{tot}}$ graph in Fig. 3 and 5.

(d) The small, potential concurrent reaction (k_{22} , Fig. 4; caption) to the final step k_{PPI} gives a value of zero within standard deviation, not justifying inclusion, see Fig. 5 and 6.





Scheme 2 “Induced fit” linkage isomerism of complex $1\text{-OP(O)}_2\text{OPO}_2(\text{OH})^{2-}$ deduced from mechanistic studies. A hypothesized octahedral elusive intermediate [3] is also depicted which was supported by computational studies.

tion in both the UV-Vis and the fluorescence kinetic studies, the spectral changes were too small and the associated relative standard deviations too large to allow for accurate analysis and was not pursued further.

However, in an attempt to further evaluate the initial substitution of the aqua ligand of complex 1-OH_2 (Scheme 2; reaction (II)), we tested a monodentate nucleophile, namely, F^- (0.16 mM) in kinetic studies at 12 °C and pH 7.35 and in the absence of PPI. These studies resulted only in a slow reaction that we assign to a background hydrolysis of the complex without significant observed coordination of the monodentate ligand to the iron center (Fig. S9†). In addition, utilization of the large excess of F^- (8.00 mM) in combination with PPI did not lead to substantial changes in the pseudo first-order rate constant of the PPI-triggered disassembly reaction of complex 1-OH_2 (Fig. S10†), although some changes in the total absorbance changes were indeed observed. This experiment suggests limited competition in the substitution of the aqua axial ligand in 1-OH_2 by F^- ions. Nevertheless, since the yield in absorbance was reduced, it indicates that there is some coordination by F^- nucleophile in a probable parallel process, potentially trapping small amounts of 1-OH_2 (Fig. S9†). However, since the experiments were performed under pseudo first-order conditions ($[\text{PPI}] \gg [1]_{\text{tot}}$), variations in $[1\text{-OH}_2]$ are not expected to influence the total kinetics observed. Moreover, as indicated for the preceding reactions with PPI (Fig. 6), small absorbance changes and large estimated standard deviations in traces with a monodentate nucleophile such as F^- ions also rendered further accurate evaluation thereof unreliable and was not pursued further.

The initial steps of the disassembly process of the PPI selective probe 1-OH_2 resemble the “induced fit” binding of a substrate to a protein during enzymatic catalysis,⁶¹ wherein the process is associated with a conformational change of the enzyme–substrate complex.^{32,61} In the disassembly of probe 1-OH_2 ,

the initial binding of $\text{HP}_2\text{O}_7^{3-}$ is followed by a switch of the monodentate ($\kappa^1\text{-O}$ pyrophosphate) to a “bidentate” coordination mode ($\kappa^2\text{-O,O'}$ pyrophosphate; Schemes 1 and 2). Based on kinetic evidence, we propose that this configurational change of the complex is associated with large conformational variations (also in agreement with the activation parameters, *vide infra*) that trigger the PPI-induced demetallation and subsequent release of signaling unit **4**.

In contrast to observations with 1-OH_2 , most activity-based probes (ABP) follow the “key-lock” principle of molecular recognition for triggering a chemical irreversible reaction after binding of the analyte.¹ We expand this concept herein to “induced fit” ABP that requires a large configurational and/or conformational change upon initial analyte binding (“induced fit” binding) for triggering the disassembly and signal transduction process.

Finally, the activation parameters of $\Delta S^\ddagger = -86 \pm 4 \text{ J K}^{-1} \text{ mol}^{-1}$ and $\Delta H^\ddagger = 62 \pm 1 \text{ kJ mol}^{-1}$ for the disassembly reaction of 1-OH_2 induced by PPI were obtained from a global fit analysis (section S1.1 and S6†) of the reactions monitored at different concentrations of $[\text{PPI}]_{\text{tot}}$ and temperatures as discussed in detail above (Fig. S2–S4 and S6†). The significant negative activation entropy associated with the final, rate-determining step is puzzling since the formation of both **4** and **5** overall might suggest a dissociative process. However, the significant structural changes required for the reorganization of intermediate [2] to [3] for triggering the disassembly (reaction (IV); Scheme 1), which in itself is a multi-step process, is assumed to be the origin of this behavior.

Conclusions

In recent years, the development of activity-based probes for biological analytes has attracted increasing attention. Despite



enormous progress, mechanistic studies on the mode of analyte detection are still rare. Herein, we report a detailed kinetic-mechanistic evaluation on the detection of PPI with an Fe-salen activity-based probe following a disassembly reaction. The data presented herein illustrate that the aqua form of the complex (**1-OH₂**) is the active species and that upon substitution of the Fe-coordinated aqua ligand and an initial end-on coordination of HP₂O₇³⁻ forms [2]. The tri-anionic pyrophosphate species is therefore selectively “trapped” following the switching from a monodentate to a bidentate coordination mode (*i.e.* linkage isomerism). The elusive intermediate [3] is further stabilized by an additional hydrogen bond, which activates the probe for the subsequent irreversible disassembly reaction and release of the fluorescent signal. The initial binding isomerism of pyrophosphate by the probe is unexpected and illustrates a structural flexible nature of the tetradentate Fe^{III}-salen complex in the presence of HP₂O₇³⁻. This unusual binding property nicely explain the previously observed outstanding selectivity of the probe for HP₂O₇³⁻. Other analytes such as orthophosphate are however potentially too small and/or lack the chelating capability (*e.g.* F⁻), or are sterically too demanding (*e.g.* ATP) for inducing the required structural changes. Alternatively, they might lack the correct geometry and flexibility coupled with appropriate hydrogen donor capability within the metal complex to trigger the disassembly of the metal-analyte complex. This also explains the inability of the di- or tetra-anions of pyrophosphate (H₂P₂O₇²⁻ and P₂O₇⁴⁻) to significantly compete in the overall reaction. It is noteworthy that the mode of initial analyte binding and recognition by the “flexible” metal complex resembles the induced-fit principle in enzymatic catalysis. We anticipate that induced fit binding of anions and molecules to “flexible” metal complexes is not restricted to the combination of pyrophosphate species and Fe-salen complexes. Therefore, we envisage that flexible metal complexes will find interesting applications in analytical chemistry, but also catalysis, medicinal chemistry, material science and related areas in the near future.

Conflicts of interest

The authors declare no competing financial interest.

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