Supporting information

Expanding the Reactivity of Inorganic Clusters towards Proteins: Interplay between Redox and Hydrolytic Activity of Ce(IV)-substituted Polyoxometalate as Artificial Protease

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1. General Remarks.

Unless otherwise noted, reactions were performed without any precautions against air and moisture. The redox reactions were performed in 1 mL solutions where the final concentrations of $Ce^{IV}K$ and Hemoglobin (Hb) were 2 and 0.02 mM, respectively. Stock solutions of $Ce^{IV}K$ (20 mM) and Hb (1 mM) were prepared in 10 mM acetate buffer (pH 5) or 10 mM phosphate buffer (pH 7.4). Reagents were purchased from commercial sources and used as received. Hemoglobin from bovine blood (Hb) was purchased from Sigma–Aldrich in the highest available purity and was used without further purification. Ce(IV)-Keggin $(Me_2NH_2)_{10}[Ce^{IV}(\alpha\text{-}PW_{11}O_{39})_2]\cdot 14H_2O$ POM ($Ce^{IV}K$), was prepared according to literature.[1]

Proton nuclear magnetic resonance (^{1}H NMR) spectra, phosphorus nuclear magnetic resonance (^{31}P NMR) and carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Bruker Avance 400 (400, 376 and 100 MHz, respectively) spectrometer. Chemical shifts (δ) for protons are reported in parts per million (ppm) downfield from tetramethylsilane proponic acid (TMSP-D_4) (0.5 mM) and are referenced to proton resonance of residual solvent peak in the NMR solvent (D_2O: δ = 4.79 ppm). ^{31}P NMR chemical shifts (δ) are reported in ppm upfield from H_3PO_4 25% (0 ppm). Chemical shifts (δ) for carbon are reported in ppm downfield from tetramethylsilane and referenced to the carbon resonances of the solvent residual peak ($CDCl_3$: δ = 77.16 ppm).

The pD value was adjusted with 1 mM NaOD and 1 mM DCl. The pH-meter reading was corrected by the equation: $pD = pH_{electrode} + 0.41$.[2] UV-Vis absorption spectra were recorded on a Varian Cary 5000 spectrophotometer and the samples were analyzed in quartz cells with a path length of 10.0 mm.

Data analysis: all NMR spectra were analyzed by using TopSpin 4.0.6. CD, UV-Vis, and tryptophan fluorescence data were processed using Origin 2018b software. 3D structure of Hb (2QSP) was prepared using Pymol and Electrostatic surfaces were calculated and visualized using the Pymol APBS plugin.[3]

2. Study of $Ce^{IV}K$ reduction in the presence of Hb by ^{31}P NMR

A 1.5 mL centrifuge tube was charged with 100 µL of 20 mM $Ce^{IV}K$ stock solution, 20 µL of 1 mM Hb stock solution, 100 µL D_2O and 780 µL buffer – 10 mM acetate buffer (pH 5.0) or 10 mM phosphate buffer (pH 7.4). The final concentration of $Ce^{IV}K$ was 2.0 nM, and of Hb was 0.02 mM, respectively. The reaction mixture was homogenized using a vortex. Next, 500 µL of the reaction mixture was transferred into an NMR tube. The reaction was monitored by ^{31}P NMR spectroscopy at different time increments and between measurements the NMR tubes were incubated at the required temperature (25, 37 or 60 °C).

A. pH 7.4 and 37 °C

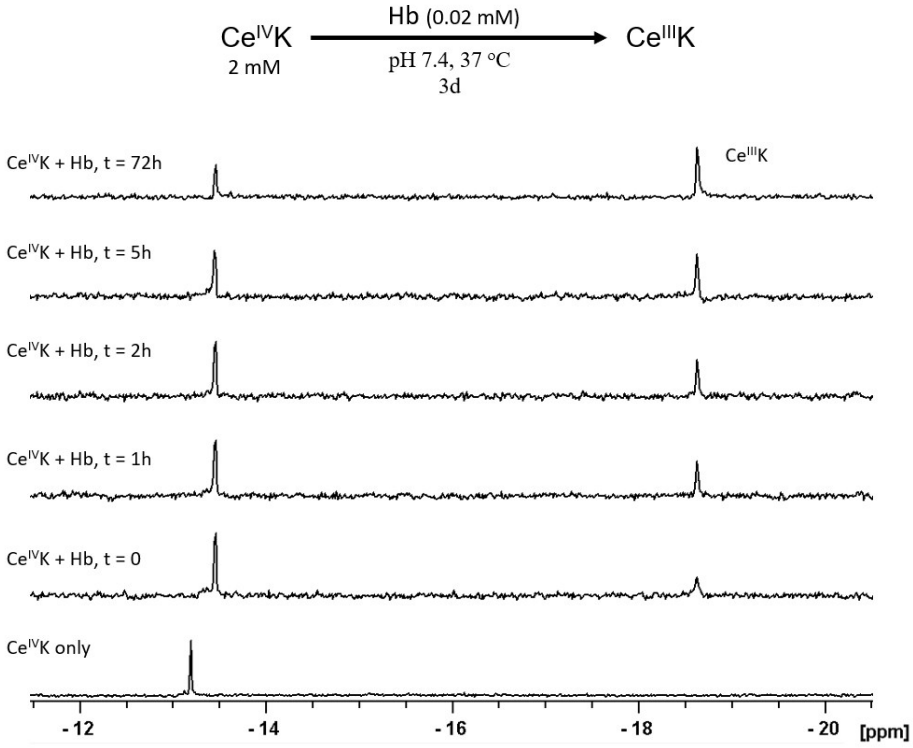


Figure S1. ^{31}P NMR spectra of the reduction of $Ce^{IV}K$ (2 mM) in the absence and presence of Hb (0.02 mM) in 10 mM phosphate buffer pH 7.4 at 37 °C.

B. pH 5.0 and 60 °C.

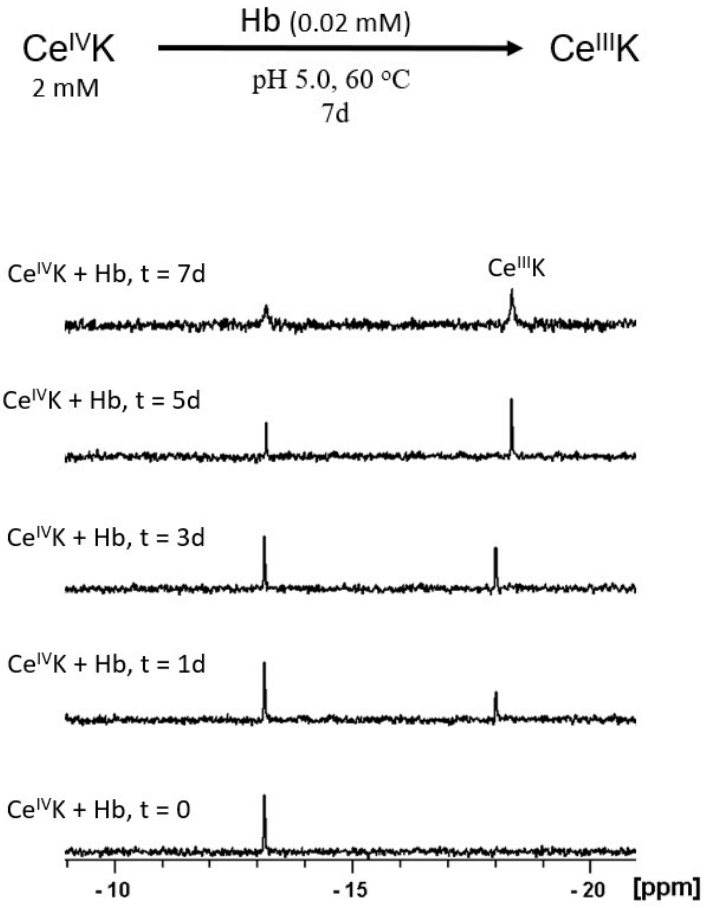


Figure S2. ^{31}P NMR spectra for the reduction of Ce^{IV}K (2 mM) in presence of Hb (0.02 mM) over 7 days, in 10 mM acetate buffer pH 5.0 at 60 °C.

3. $Ce^{IV}K$-Hb interaction studies via Tryptophan fluorescence spectroscopy

Solutions containing Hb (10.0 µM) and $Ce^{IV}K$ (0 to 15.0 µM) were prepared in 10.0 mM acetate buffer (pH 5.0) or 10.0 mM phosphate buffer (pH 7.4). Emission fluorescence spectra were recorded on a Photon Technology Quanta Master QM-6/2005 spectrofluorometer. Quartz cuvettes with 10.0 mm optical path length were used. Spectra were recorded at room temperature. The emission was monitored from 300 nm to 420 nm. Sample was irradiated with a wavelength of 295 nm to avoid excitation of tyrosine residues. The emission and excitation slit widths were opened at 0.74 mm (resolution of 2.0 nm).

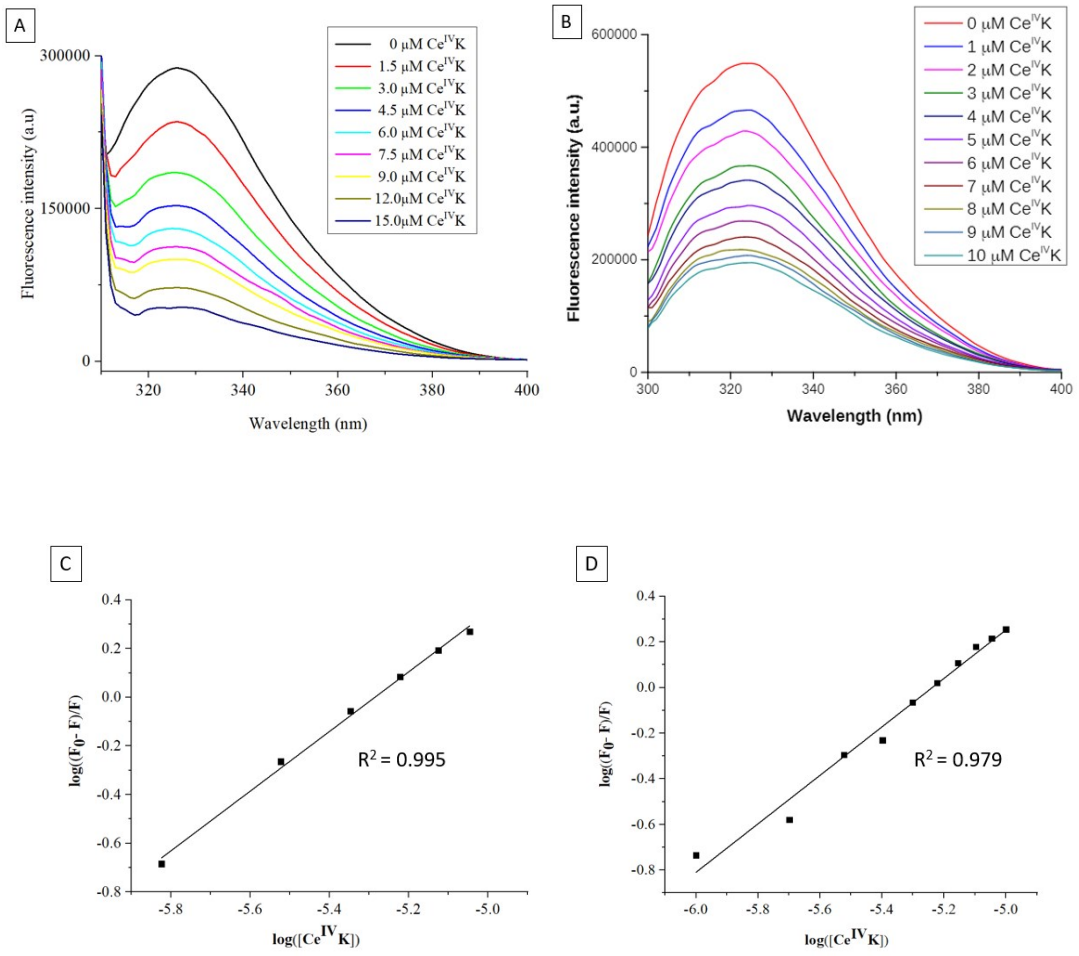


Figure S3. Spectra of tryptophan fluorescence quenching of Hb (10.0 µM) in the presence of different $Ce^{IV}K$ concentration at 25 °C at (A) pH 5.0 and (B) pH 7.4, and their corresponding Stern–Volmer plots for quenching observed at (C) pH 5.0; (D) pH 7.4.

4. Effect of $Ce^{IV}K$ on Hb secondary structure

A. ^{1}H NMR spectroscopy.

Solutions containing Hb (0.5 mM) in the absence or presence of 10% sodium dodecyl sulphate (SDS) and 0.1 M dithiothreitol (DTT) or 0.25 mM $Ce^{IV}K$ were prepared in 10.0 mM acetate buffer (pH 5.0, 10% D_2O). ^{1}H NMR spectra of Hb were recorded at room temperature directly after sample preparation (Figure S4).

Protein folding can be monitored by recording the ^{1}H NMR spectra since the resonances for the amide group between $\delta = 6 - 10$ ppm, and for aliphatic amino acid side chains $\delta < 0$ ppm are characteristic features of a folded protein structure. As can be seen from Figure S4, these characteristic signal from the native protein remained unchanged in the presence of $Ce^{IV}K$, indicating that Hb largely conserves its folded structure in the presence of $Ce^{IV}K$. Such conclusion is further confirmed by comparing the ^{1}H NMR in the presence of $Ce^{IV}K$ with the one in the presence of a denaturant (10% SDS and DTT) known to unfold the protein, in which the above mentioned resonances are not present.

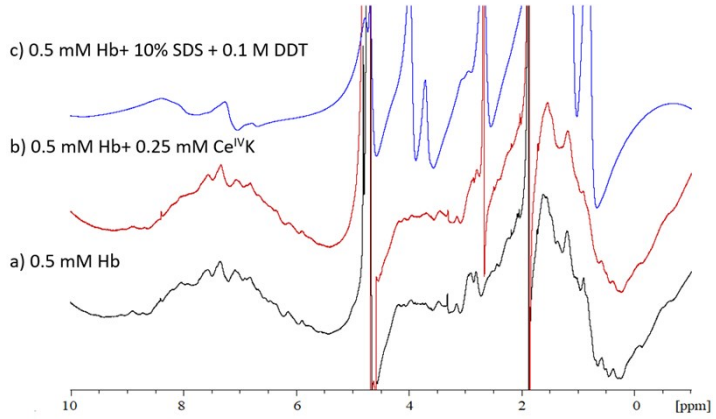


Figure S4. ^{1}H NMR spectra of Hb in absence and presence of $Ce^{IV}K$ or SDS/ DDT.

B. UV-Vis spectroscopy.

Solutions containing Hb (2.0 or 10.0 µM) and $Ce^{IV}K$ (0 to 50 0r 100 µM) were prepared in 10 mM acetate buffer pH 5.0 or 10 mM phosphate buffer pH 7.4. The UV-Vis absorption spectra of Hb with and without SDS in 10 mM acetate buffer pH 5.0 are showed in Figure S5-A. In the absence of SDS, the spectrum shows the Soret band at 405 nm, which corresponds to the met-conformational structure of Hb. The addition of SDS caused a marked decrease in the intensity in the maximum of the Soret band. In addition, broad and blue shift (from 405 to 370 nm) in the Soret band were also observed. These features are consequences of unfolding of Hb in the presence of a denaturant (SDS). However, in presence of different concentrations of $Ce^{IV}K$, (0 - 50µM), a slight red shift could be detected (Figure S5-B). Additionally, the intensity of the Soret band decreased gradually upon increasing in the $Ce^{IV}K$ concentration, indicating an interaction of $Ce^{IV}K$ with Hb.

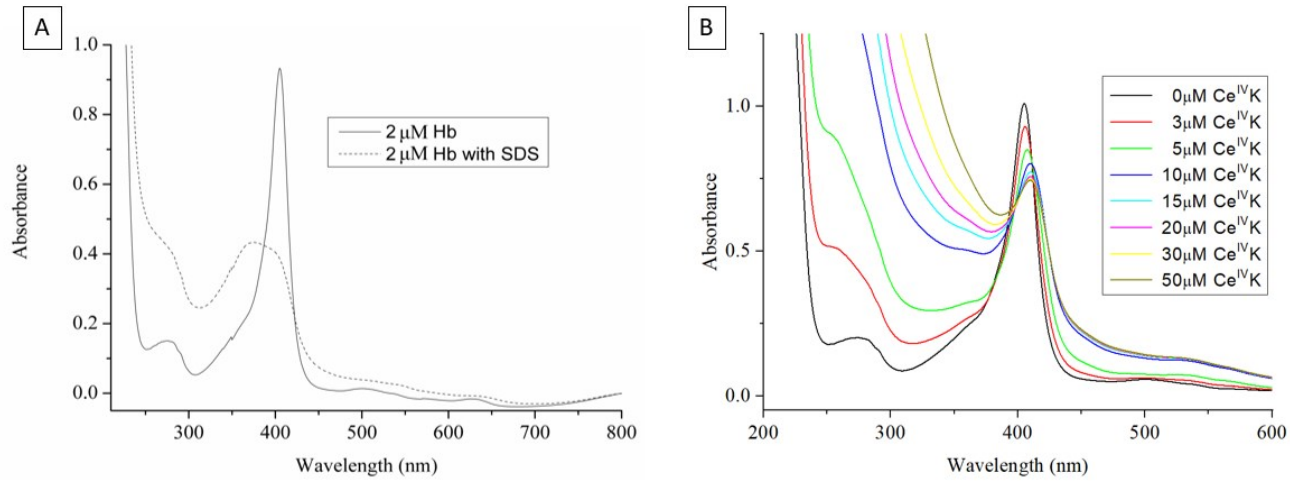


Figure S5. UV-Vis absorbance spectra of Hb; A) Hb (solid line) and Hb + SDS (dotted line). B) Hb (2.0 μM) in the presence $Ce^{IV}K$ (0 – 50 μM).

C. CD spectroscopy.

Solutions containing Hb (5.0 μM) and complexes $Ce^{IV}K$ (0 to 15.0 μM) were prepared in 10 mM acetate buffer pH 5.0. CD measurements were performed by using a JASCO J-810 spectropolarimeter. Quartz cuvettes with 1.0 mm optical path length were used. Far-UV wavelength scans were recorded from 180 to 260 nm. Exclusion of the background effect for all measurements was done by subtracting the spectrum of the respective buffer solution from the spectrum of the protein. The CD spectra in the presence of increasing amounts of $Ce^{IV}K$ is presented in Figure S6. The results strongly confirmed an interaction of $Ce^{IV}K$ with Hb as a decrease in ellipticity values (l=208 and 222 nm) with the increase of $Ce^{IV}K$ concentration was observed.

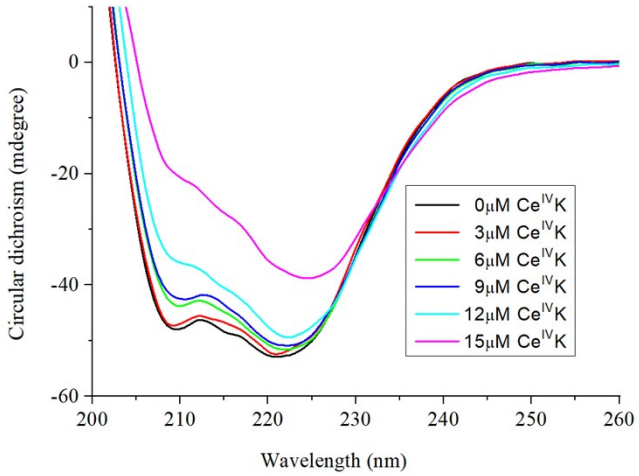


Figure S6. CD spectra evidence changes in Hb (10.0 μM) secondary structure in the presence of $Ce^{IV}K$ (0 – 15 μM) at 10 mM acetate buffer pH 5.0 and 25 °C.

5. Hemoglobin Hydrolysis

A. Sample preparation.

Solutions containing bovine Hb (0.02 mM) and $Ce^{IV}K$, $Ce^{III}K$ or $Na_9[PW_9O_{34}]$ (2.0 mM) were prepared in 10.0 mM acetate buffer (pH 5.0) or 10.0 mM phosphate buffer (pH 7.4). Samples were incubated at 37 °C or 60 °C. Aliquots were taken at different time increments and analyzed by SDS-PAGE.

B. Electrophoresis.

SDS-Tricine-PAGE was performed on a 5% (w/v) polyacrylamide gel in 3.0 M Tris-HCl buffer pH 8.45 stacking gel and a 18% (w/v) polyacrylamide in 3.0 M Tris-HCl buffer pH 8.45 resolving gel. Samples (15 µL) were supplemented with 5 µL sample buffer and heated at 100 °C for 5 min, followed by loading 10 µL of the resulting solution on the gel. Unstained low range protein ladder (3.4 to 100 kDa) was used as a molecular mass standard. An OmniPAGE electrophoretic cell was combined with an EV243 power supply (both produced by Consort, Turnhout, Belgium). Experiments were performed at 200 V for 2.0 h. Proteins in SDS-Tricine-PAGE gels were visualized with silver staining and an image of each gel was taken using a GelDoc EZ Imager (Bio-Rad, Hercules, CA).

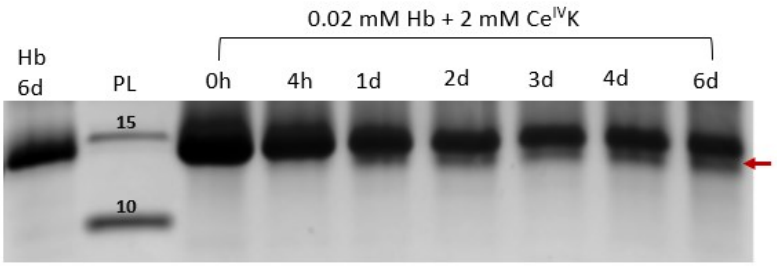


Figure S7. SDS-PAGE gels for the hydrolysis of Hb (0.02 mM) in presence of $Ce^{IV}K$ (2 mM) at pH 7.4 and 60 °C. Red arrow indicates the hydrolytic fragment at 14.0 kDa.

C. Identification of fragments by Edman degradation and LC-MS/MS.

SDS-PAGE gels were blotted onto a PVDF membrane and stained with Coomassie blue. The bands were cut from the membrane destained with methanol and rinsed with ultrapure water. The bands were subjected to automated N-terminal amino acid sequence analysis (Procise 491 cLC protein sequencer, Applied Biosystems, Foster City, CA) based on the Edman degradation reaction as described in the literature.[4] Alternatively, after the hydrolysis proteins and peptide fragments were purified by reversed-phase UPLC on an UltiMate 3000 RSLC nano system (Thermo Scientific, Waltham, MA)

coupled to an Amazon ETD ion trap mass spectrometer (Bruker, Bremen, Germany) for MS/MS analysis. Samples were filtered through 0.2µm filters (Minisart RC4, Sartorius Stedim Biotech, Goettingen, Germany), the filtrate centrifuged at 10000 g for 10 minutes prior to injection at a flow rate of 3 µL/min on a Acclaim PepMap RSLC C18 column (75µm x 15cm; Thermo Scientific) equilibrated in 0.1% (v/v) trifluoroacetic acid with 4% (v/v) acetonitrile. Peptides were eluted in an acetonitrile gradient in 0.1% (v/v) formic MS/MS and collision-induced dissociation spectra were collected.

Table S1. Overview of N-terminal amino acid sequences for Hb hydrolytic products induced by $Ce^{IV}K$ determined by Edman degradation.

Sequence determined	Fragment	Mol. Mass (kDa)	Peptide bond cleaved
LPGALSELSDLHA	α(Leu76-Arg141)	7.2	α(**Asp**75-Leu76)
PVNFKLLSHSLLVTL	α(Pro95-Arg141)	5.1	α(**Asp**94-Pro95)
AVMNNPKVKA	β(Ala52-His145)	10.3	β(**Asp**51-Ala52)
SFSNGMK	β(Ser69-His145)	8.5	β(**Asp**68-Ser69)
DLKGTFAALSELH	β(Asp79-His145)	10.9	β(**Asp**78-Asp79)
PENFKLLGNVLVVVL	β(Pro99-His145)	5.1	β(**Asp**98-Pro99)
MLTAEEKAAV	β(Met1-Asp128)	14.0	β(**Asp**128-Phe129)

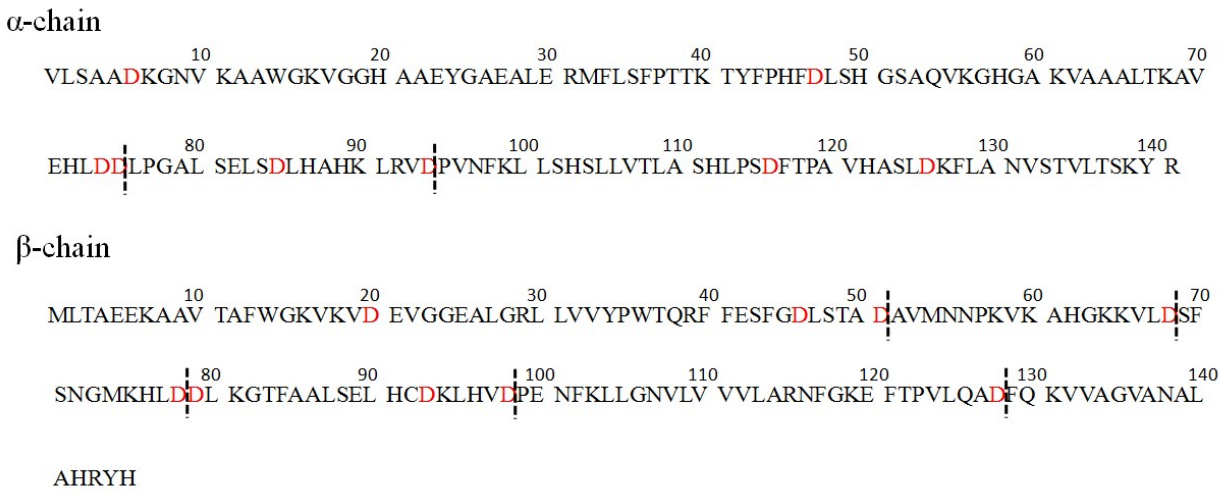


Figure S8. Primary amino acids sequence of Hb with the corresponding cleavage sites obtained in the presence of $Ce^{IV}K$. Asp residues are in red.

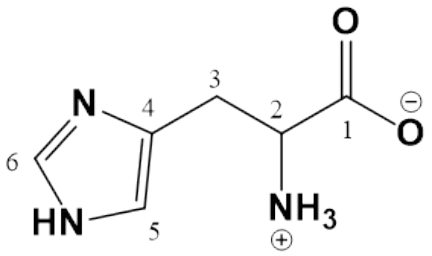
Table S2. Difference between $Ce^{IV}K$ and 2:2 ZrK^5 cleavage sites of Hb.

Hb chain	$Ce^{IV}K$	ZrK
α-chain	α(**Asp**75-Leu76) α(**Asp**94-Pro95)	α(**Asp6**-Lys7) α(**Asp74**-Asp75) α(**Asp**75-Leu76) α(**Asp**85-Leu86) α(**Asp**94-Pro95) α(**Asp**116-Phe117)
β-chain	β(**Asp**51-Ala52) β(**Asp**68-Ser69) β(**Asp**78-Asp79) β(**Asp**98-Pro99) β(**Asp**128-Phe129)	β(**Asp**46-Phe47) β(**Asp**51-Ala52) β(**Asp**68-Ser69) β(**Asp**78-Asp79) β(**Asp**98-Pro99)

6. Interactions of $Ce^{IV}K$ with His

A 1.5 mL centrifuge tube was charged with (0.124 g ,20 mM) $Ce^{IV}K$ and 900 µL of D_2O, the mixture was vortexed until complete dissolution (Solution **A**). Solution **A** (900 µL) was transferred to another 1.5 mL centrifuge tube charged with (0.0031 g, 20 mM) His. after mixing, 100 µL of D_2O was added. The reaction mixture was homogenized using a vortex. Next, as an internal standard, 0.5 mM TMSP-d_4 was used for ^1H NMR and 1% TMS in $CDCl_3$ was used as ^{13}C NMR external standard. 500 µL of the reaction mixture was transferred to an NMR tube. All measurements were done at room temperature on a Bruker Avance 400 spectrometer. The measurements were performed immediately after mixing.

Table S3. ^1H and ^{13}C NMR chemical shift values of 20 mM His in presence and absence of 20 mM $Ce^{IV}K$



Atom	20 mM His	20 mM His + 20 mM $Ce^{IV}K$	Δδ (ppm)
C1	174.11	173.58	- 0.53
C2	54.92	54.54	- 0.38
C3	28.31	27.44	- 0.87
C4	132.35	130.52	- 1.84
C5	116.82	117.31	0.49
C6	136.46	135.79	- 0.67
$H_{(C3)}$	3.1-3.2	3.18-3.35	0. 13
$H_{(C2)}$	3.94-3.97	4.01-4.04	0.072
$H_{(C5)}$	7.02	7.22	0.2
$H_{(C6)}$	7.72	8.06	0.34

7. References.

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