Comparative studies of soluble and immobilized Fe(III) heme-peptide complexes as alternative heterogeneous biocatalysts

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Supplementary material

Text S1

Heme-peptide binding studies

Determination of binding affinity (K_D) and number of independent binding sites (n) in binding studies for Fe(III) heme to a peptide was performed as described before (Syllwasschy et al. 2020). Hemin (1 mM) was dissolved in 30 mM NaOH and diluted with 0.1 M Hepes (pH 7.0) prior to usage. Peptides were dissolved in 0.1 M Hepes and mixed with Fe(III) heme at a constant peptide concentration (10 or 20 µM) and varying Fe(III) heme concentrations (0.4-60 µM). After 30 min incubation, heme-peptide complexes have been measured by UV/Vis-spectroscopy. Difference spectra were generated by subtraction of the corresponding spectrum for heme only at each concentration. Determination of K_D and n was performed by the following equation according to Pîrnau et al. (Pîrnau and Bogdan 2008):

$$\Delta A = 0.5 \times \Delta\varepsilon \times ((C_H + n \times C_P + K_D) \pm ((C_H + n \times C_P + K_D)^2 - 4 \times C_H \times n \times C_P)^{0.5})$$

(ΔA – difference in absorbance at the wavelength of the maximum in the difference spectrum; $\Delta\varepsilon$ – difference in the molar extinction coefficients of Fe(III) heme and the Fe(III) heme-peptide complex; C_H – Fe(III) heme concentration; n – number of independent Fe(III) heme binding sites on the peptide, C_P – peptide concentration; K_D – dissociation constant)

For analysis of Fe(III) heme binding to peptide at pH 5.0, the Fe(III) heme-peptide complex (40 µM) was prepared as described above and diluted 1:1 with 0.1 M Hepes (pH 7.0) or 0.5 M citrate buffer (pH 5.0). Subsequently, the Fe(III) heme-peptide complexes were measured by UV/Vis spectroscopy.

All data have been evaluated using PRISM 9 (GraphPad Software).

Text S2

Steady-state kinetics for substrate conversion

For determination of kinetic parameters of soluble complexes, initially Fe(III) heme-peptide complexes (21 µM, 1:1) were prepared as described in Text S1 in Hepes (0.1 M, pH 7.0) and incubated for 30 min. Subsequently, they were added to a mixture of substrates TMB and H_2O_2 in citrate buffer

(0.1 M, pH 5.0) to yield a final complex concentration of 1 µM. TMB and H_2O_2 were applied in varying concentrations as stated in the corresponding figures and tables. Oxidation of TMB was followed at 652 nm (ε_{652} = 3.9 × 10^4 M^{-1} cm^{-1}). Data were fit to the Michaelis-Menten equation or Lineweaver-Burk double reciprocal equation assuming saturation of the catalyst with the non-changed substrate.

For analysis of steady-state kinetics of immobilized complexes, the resin-bound peptide was washed in PBS buffer (pH 7.4, 0.01% w/v Tween 20) for 5 × 1 min and 1 × 60 min in a 0.8 ml column (Biospin, Bio-Rad). Subsequently, the resin was washed for 1 min with *dd*H_2O and incubated with 1 µM Fe(III) heme in PBS buffer (pH 7.4, 0.01% w/v Tween 20) for 30 min. Hemin stock solution (1 mM) was prepared in 30 mM NaOH as mentioned in Text S1 and diluted in PBS buffer (pH 7.4, 0.01% w/v Tween 20) as required. After the incubation of the resin with Fe(III) heme, the solution was aspirated and the resin was washed for 3 min with *dd*H_2O followed by addition of the substrate mixture containing TMB and H_2O_2 in citrate buffer (0.1 M, pH 5.0) at varying concentrations as stated in the corresponding figures and tables and measured at specific intervals for reaction rate determination. Oxidation of TMB was followed at 652 nm (ε_{652} = 3.9 × 10^4 M^{-1} cm^{-1}). Data were fit to the Michaelis-Menten equation assuming saturation of the catalyst with the non-changed substrate.

All data have been evaluated using PRISM 9 (GraphPad Software).

Table S1. Analytical characterization of peptides derived by truncation of 23mer peptide 1.

No.	Sequence[a]	M_W (M_W theor.) [g/mol][b]	HPLC t_R [min][e]	TLC R_f[f]
1	NVNLTSNHLLYHYWIAVSHKAPA	1324.43[c] (2646.38)	18.1	0.07[g] 0.34[h]
2	VNLTSNHLLYHYWIAVSHKAP	1231.65[c] (2461.30)	18.9	0.09[g] 0.36[h]
3	NLTSNHLLYHYWIAVSHKA	1133.60[c] (2265.18)	18.5	0.10[g] 0.31[h]
4	LTSNHLLYHYWIAVSHK	1041.05[c] (2080.10)	17.2	0.12[i] 0.36[h]
5	TSNHLLYHYWIAVSH	920.46[c] (1838.92)	17.5	0.19[i] 0.36[h]
6	SNHLLYHYWIAVS	801.42[c] (1600.02)	17.4	0.31[i] 0.40[h]
7	NHLLYHYWIAV	714.38[c] (1426.75)	17.5	0.36[i] 0.51[h]
8	HLLYHYWIA	1214.64[d] (1213.64)	15.2	0.49[g] 0.49[i]

[a]Peptides were synthesized as C-terminal amides as described earlier (Kühl et al. 2011; Wißbrock et al. 2017). All peptides were >95 % HPLC pure. [b]Data for mass spectrometry were collected with an LC-ESI micrOTOF-Q III system (Bruker Daltonics GmbH) coupled to a Dionex Ultimate 3000 LC (Thermo Fisher Scientific) as described earlier (Wißbrock et al. 2017). Mass peaks were detected as [c]$[M+2H]^{2+}$ and [d]$[M+H]^{+}$. [e]HPLC conditions were as follows: water with 0.1 % TFA (eluent A), acetonitrile with 0.1 % TFA (eluent B), and a gradient was run for all samples from 20%-50% eluent B in 30 min (flow rate: 1 ml/min; UV detection at 220 nm). The column was a C18 Vydac 218TP54 with 4.6 x 25 mm, 5 mm particle size, and 300 Å pore size. [f]TLC was performed on silica-coated glass plates using the following systems: [h]n-propanol/25 % ammonia (3:7, v/v), [g]pyridine/ethyl acetate/acetic acid/water (5:5:1:3, v/v), [i]n-butanol/acetic acid/water (48:18:24, v/v). Detection was performed by staining with ninhydrin or potassium iodide containing o-tolidine reagent (after treatment with chlorine for several minutes and subsequent chlorine evaporation).

Figure S1. Difference absorbance spectra of peptides 1-7. Spectra were prepared by measurement of constant peptide concentrations (10 or 20 µM) and varying Fe(III) heme concentrations (0.4-60 µM) after 30 min incubation in Hepes buffer (0.1 M, pH 7.0). Difference spectra were prepared by subtraction of the spectrum for pure Fe(III) heme from the incubated Fe(III) heme-peptide complex at the same Fe(III) heme concentration. The maximum of the Soret band for each complex is designated in the corresponding set of difference spectra for each peptide and indicated by an arrow. Inset: Zoom-in between 500 and 600 nm for the difference spectrum produced with the highest Fe(III) heme concentration for the corresponding peptide. Annotated are α and β bands.

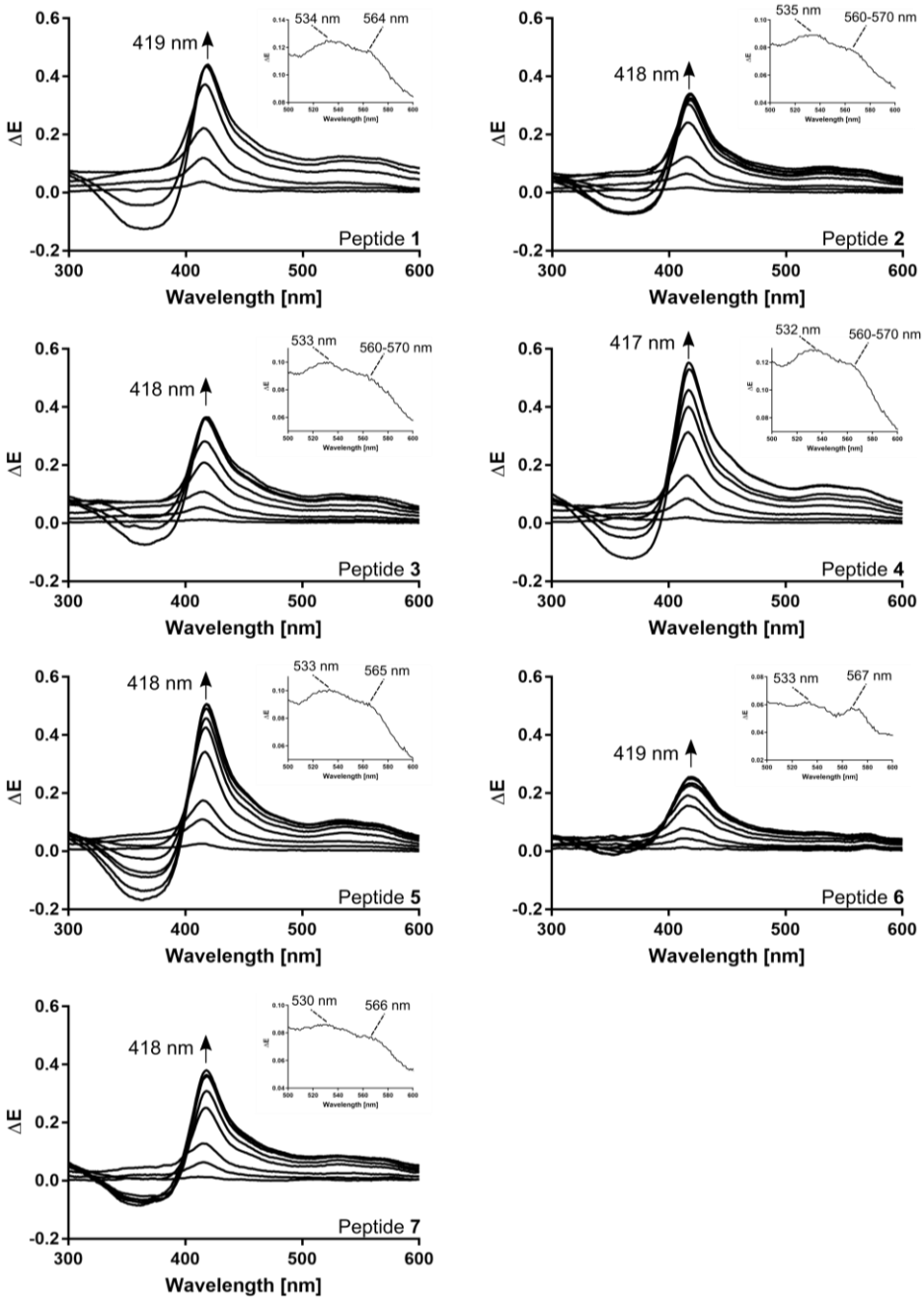


Figure S2. Absorbance spectra of Fe(III) heme-peptide complexes at different pH values. UV/Vis spectra were prepared by mixing peptide and Fe(III) heme (1:1, 40 µM) in Hepes buffer (0.1 M, pH 7.0) and dilution (1:1) after 30 min incubation into Hepes buffer (0.1 M, pH 7.0) or citrate buffer (0.5 M, pH 5.0). Final Fe(III) heme-peptide complex concentration was 20 µM at pH 7.0 or pH 5.0. Inset: Zoom-in between 500 and 600 nm for the absorbance spectrum for the corresponding peptide at different pH values.

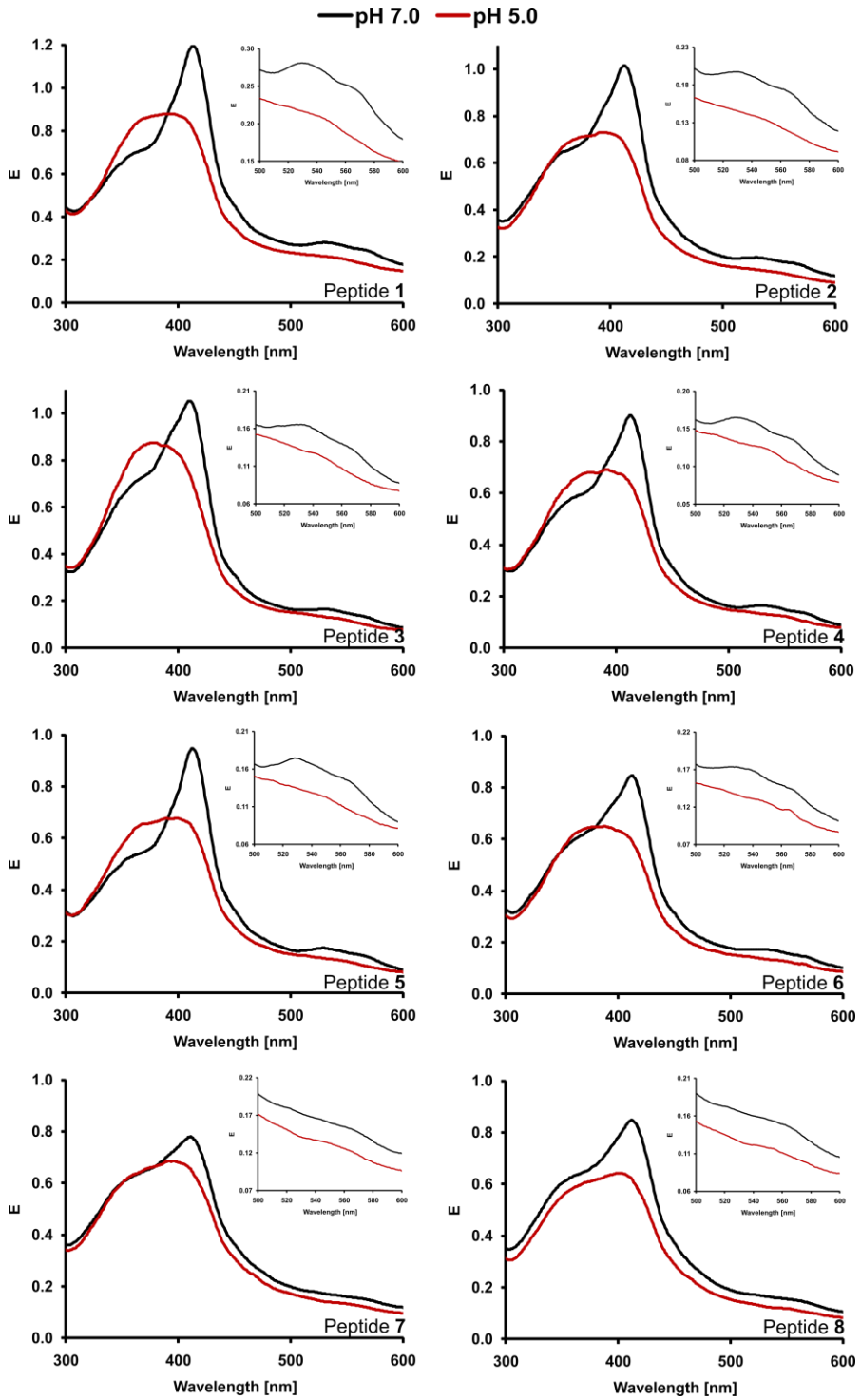
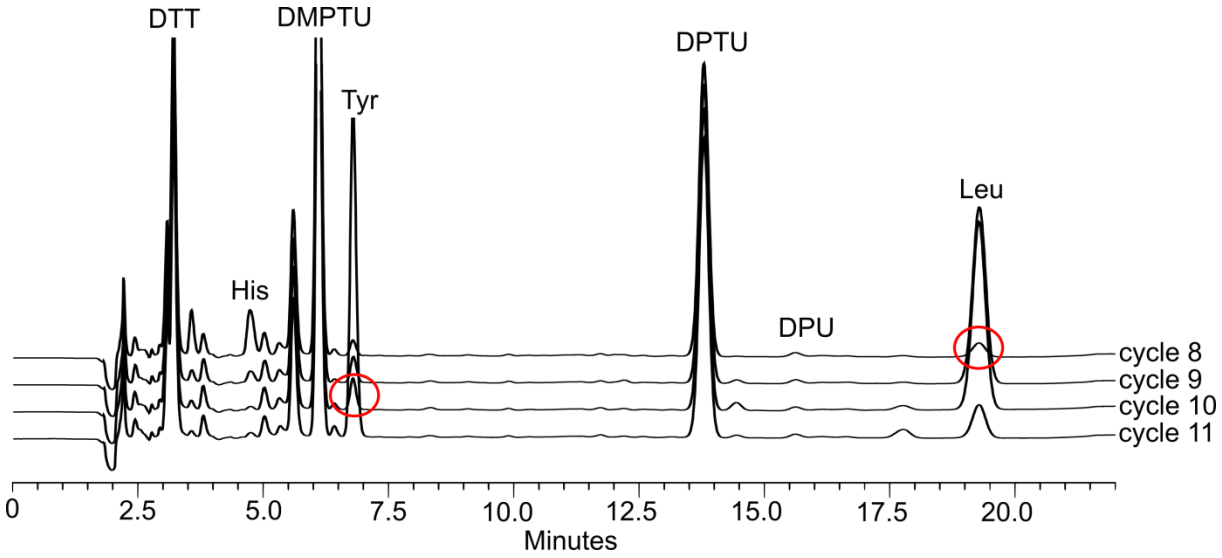


Figure S3. Edman degradation of peptide 1 synthesized on Tentagel-S-NH_2 resin. Cycle runs of N-terminal sequencing covering amino acid positions 8-11 of one bead of immobilized peptide **1** on a PPSQ-53A (Shimadzu). An increase in the intensity of the correlating phenylthiohydantoin (PTH)-derivatives can be observed that is representative for the occurrence of amino acids histidine, leucine, leucine, and tyrosine, respectively. A minor amount of PTH-leucine in cycle 8 as well as PTH-tyrosine in cycle 10 indicates a small fraction of wrongly synthesized peptide **1** missing histidine or leucine in the same position. As is known for the method, occurrence of residual PTH-amino acids from preceding cycles that were not cleaved in that cycle can be still detected in the presented cycles. The total run for this peptide was performed for 23 cycles confirming the existence of the peptide sequence NVNLTSNHLLYHYWIAVSHKAPA.



References

Pîrnau, A., Bogdan, M. (2008) Investigation of the interaction between naproxen and human serum albumin. Rom. J. Biophys. 18: 49–55.

Syllwasschy, B.F., Beck, M.S., Družeta, I., Hopp, M.-T., Ramoji, A., Neugebauer, U., Nozinovic, S., Menche, D., Willbold, D., Ohlenschläger, O., Kühl, T., Imhof, D. (2020). High-affinity binding and catalytic activity of His/Tyr-based sequences: Extending heme-regulatory motifs beyond CP. Biochim. Biophys. Acta Gen. Subj. 1864(7): 129603.