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Synthesis and Characterization of Metal Binding Pseudotripeptides

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Abstract: Metal complexes with peptide or pseudopeptide type ligands can serve as good model compounds for a deeper understanding of enzymatic catalysis, but ligands with a high selectivity for different transition metal cations are hard to find due to the rather flexible nature of peptides. Since such ligands would be the *sine qua non* condition for the synthesis of heterodinuclear peptide metal complexes with catalytic activity, the search for small, affine and selective metal chelating sequences is of interest. Using four different amino acids (His, Lys, Asp, Glu) a set of 16 pseudotripeptides of the common structure Bz-AS^1-Sar-AS^2-NH_2 has been synthesized, purified and characterized by mass spectrometry and ^{1}H-NMR. Their ability to form metal complexes has been investigated leading to short motifs capable of selectively binding only one or two transition metal cations with high affinity. As expected, the complexation of transition metal cations by pseudotripeptides is strongly dependent not only on the amino acid composition, but also on the sequence with regard to the stability of the resulting complexes, as well as the selectivity of the ligands towards Cu^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} and Mn^{2+}.

Keywords: metal complex; peptide ligands; pseudopeptides

INTRODUCTION

Transition metal cations are essential to the function of many enzymes, such as structure related functions in zinc finger motifs or direct involvement in the catalytic cycle as in metalloproteases or many oxidoreductases. One approach to a deeper understanding of the functional, i.e. catalytic, properties of such enzymes is the search for appropriate models, that, while having a measurable catalytic activity, would be smaller and thus easier to study. Such bioinorganic enzyme models have been published for a wide variety of enzymes [1], ranging from carbonic anhydrase models [2,3] to mimics of oxidoreductases such as lipoxygenase [4] or catechol oxidase [5]. The superoxide dismutase mimic M40403, described by Daniela Salvemini and coworkers, could even be shown to have enzyme like activity and clinical importance [6].

Chiral catalysts deserve special attention, since they are capable of performing asymmetric reactions [7–10] by forming diastereomeric transition states with prochiral or racemic substrates, making them potentially interesting for commercial applications. Enzymes achieve unmatched enantioselectivity by the same base mechanism, they are built from chiral monomers, the amino acids, so a rather obvious approach to the synthesis of enzyme mimics would

Abbreviations: AA, amino acid; DIEA, diisopropylethylamine; DMF, N,N-dimethylformamide; ESI, electro spray ionization; Fmoc, fluorenylmethyloxycarbonyl; HPLC, high performance liquid chromatography; MALDI-TOF, matrix assisted laser desorption ionization — time of flight; MS, mass spectrometry; NMR, nuclear magnetic resonance; PyBOP®, benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate; TIS, triisopropylsilane; TFA, trifluoroacetic acid.

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be the use of peptide or peptoid ligands, which has been done extensively [11–14].

Greiner and coworkers have described a set of metal chelating pseudotripeptides incorporating N-functionalized glycine residues of the common structure Bz-His-N[R]Gly-His-NH_2 [15]. However, these pseudotripeptides did not differ much in terms of selectivity [16]. In our attempt to synthesize heterodinuclear complexes with peptoid ligands, it was decided to replace systematically the two histidine residues by all possible combinations of histidine, lysine, aspartic acid and glutamic acid while keeping the *N*-functionalized glycine as small as possible, i.e. incorporating sarcosine, leading to pseudotripeptides of the common structure Bz-AA^1-Sar-AA^2-NH_2. Some of them show good affinity towards transition metal ions and a high selectivity for only one or two of them, making them possibly useful modules for studying preferred binding motifs for certain metal ions and for the design of mimetics for enzymes with a heterodinuclear metal core. The synthesis and chelating properties of these ligands are presented, along with the methods used for their determination. Based on these studies we are currently trying to elucidate the relationship between the structure of pseudopeptide metal complexes and their catalytic activities and mechanisms of catalysis.

MATERIALS AND METHODS

General

Chemicals and metal salts were purchased from Aldrich, Fluka and Alfa Aesar. Amino acid derivatives and PyBOP® were purchased from Nova-Biochem (Switzerland). All solvents were purified by distillation.

Synthesis and Purification of Pseudotripeptides

All compounds were synthesized in reaction syringes (ABIMED), using Rink Amide MBHA resin (Nova-Biochem, Switzerland) with a maximum loading of 0.73 mmol/g. Resin loading, deprotection and coupling was performed according to standard procedures of Fmoc-based peptide synthesis, no special solvents and coupling reagents, other than DMF and PyBOP®, were required. Fmoc was removed by treatment with 20% piperidine in DMF for 5 and 15 min and subsequently washing ten times with DMF. Single coupling (2 h) with 2.5 eq PyBOP®, 2.5 eq Fmoc-amino acid and 5 eq DIEA in DMF proved to be sufficient according to the Kaiser-test in all cases. Final benzoylation of the tripeptides was done by adding a solution of 10 eq of benzoic anhydride and 15 eq of DIEA (diisopropylethylamine) in 2 ml of DMF to the reaction vessel and shaking for 3 h. The procedure was then repeated with a fresh solution for another hour.

Cleavage from the resin and of side chain protecting groups was performed simultaneously by adding 2.5% TIS (triisopropylsilane), 2.5% water and 95% TFA (trifluoroacetic acid) to a volume of 5 ml/g of resin. The mixture was shaken for 4 h and the filtrate was added dropwise to 50 ml of cold diethylether, where precipitation occurred for all peptides, except for those containing two acidic residues (Table 1). The precipitated peptides were filtered and washed with ether, the solutions of the soluble peptides were concentrated *in vacuo*. Finally all the compounds were dissolved in 80% tert-butyl alcohol and lyophilized.

The lyophilized compounds were purified by semi-preparative HPLC (LC-8A, Shimadzu) on a Knauer RP18 C5 column with a diameter of 32 mm and a length of 250 mm. Water with 0.1% TFA (system A) and 90% acetonitrile with 0.1% TFA (system B) were used as eluent in a gradient system from 0% system B to 50% system B in 120 min. The purity of fractions was checked by analytical HPLC (LC 10AT, Shimadzu) on a Vydac 218TP C5 column with a diameter of 4.6 mm and a length of 25 mm using a gradient system from 0% system B to 60% system B in 60 min. Detection in both cases was done by measuring absorption at 220 nm wavelength. The purified fractions were combined and lyophilized. Identity was checked using MALDI-TOF mass spectrometry and ^{1}H-NMR (250 MHz, Bruker Instruments). All ^{1}H-NMR spectra were recorded in DMSO-D6 at a concentration of 10 mg/ml with 128 scans. Ion exchange (only TP2) was done using Amberlite IRA 410 (Serva) ion exchange resin.

TP1: Bz-His-Sar-His-NH_2 × 2 TFA. ^{1}H-NMR: $\delta = 14.20$ (s, 4H, $NH_{im.prot.}$, $His^{1/3}$), 7.39–8.96 (m, 11H, CH benzoyl, *C*-term. amide $CONH_2$, $CH_{im.prot.}$, $His^{1/3}$), 7.27–7.32 (m, 2H, backbone NH), 5.14–5.24 (m, 1H, $C_\alpha H$, His^1), 4.39–4.51 (m, 1H, $C_\alpha H$, His^3), 3.86–4.12 (m, 2H, $C_\alpha H_2$, Sar), 2.69–3.15 (m, 7H, NCH_3, Sar, $C_\beta H_2$, $His^{1/3}$).

Mass calculated: 466.21, found: 467.1 $[M + H]^+$; HPLC retention time: 16.4 min.

Table 1 Pseudotripeptides and their Properties

No.	*N*-term	Aa^1	Aa^2	Aa^3	*C*-term	Selectivity	K_{diss} Cu^{2+} (μM)
1	Bz	His	Sar	His	NH2	Cu ~ Ni > Zn ~ Co > Mn	<0.1
2	Bz	Lys	Sar	His	NH2	Cu > Ni > Zn ~ Co > Mn	37
3	Bz	Asp	Sar	His	NH2	Cu ~ Co ~ Mn > Ni ~ Zn	0.3
4	Bz	Glu	Sar	His	NH2	Ni ~ Cu > Co > Zn ~ Mn	10
5	Bz	His	Sar	Lys	NH2	Cu > Co ~ Ni > Zn > Mn	11
6	Bz	Lys	Sar	Lys	NH2	No complexation	114
7	Bz	Asp	Sar	Lys	NH2	No complexation	197
8	Bz	Glu	Sar	Lys	NH2	No complexation	240
9	Bz	His	Sar	Asp	NH2	Co ~ Ni > Zn, Cu, Mn	13
10	Bz	Lys	Sar	Asp	NH2	No complexation	118
11	Bz	Asp	Sar	Asp	NH2	No complexation	228
12	Bz	Glu	Sar	Asp	NH2	No complexation	94
13	Bz	His	Sar	Glu	NH2	Co ~ Ni > Zn > Cu ~ Mn	148
14	Bz	Lys	Sar	Glu	NH2	No complexation	394
15	Bz	Asp	Sar	Glu	NH2	No complexation	502
16	Bz	Glu	Sar	Glu	NH2	No complexation	374

TP2: Bz-Lys-Sar-His-NH$_2$. ^{1}H-NMR: $\delta = 7.42–8.65$ (m, 9H, CH benzoyl, *C*-term. amide $CONH_2$, $CH_{im.}$, His), 6.74–7.28 (m, 3H, backbone NH, $NH_{im.}$, His), 4.72–4.89 (m, 1H, $C_\alpha H$, Lys), 4.40–4.47 (m, 1H, $C_\alpha H$, His), 3.76–4.16 (m, 2H, $C_\alpha H_2$, Sar), 2.67–3.08 (m, 7H, NCH_3, Sar, $C_\beta H_2$, His, $C_\varepsilon H_2$, Lys), 1.84 (s, 2H, $C_\varepsilon NH_2$, Lys), 1.38–1.70 (m, 6H, $C_{\beta,\gamma,\delta}H_2$, Lys).

Mass calculated: 457.24, found: 457.7 $[M + H]^+$; HPLC retention time: 16.7 min.

TP3: Bz-Asp-Sar-His-NH$_2$ × TFA. ^{1}H-NMR: $\delta = 14.12$ (s, 2H, $NH_{im.prot.}$, His), 12.35 (s, 1H, $C_\gamma OOH$, Asp), 7.26–8.97 (m, 11H, CH benzoyl, *C*-term. amide $CONH_2$, $CH_{im.prot.}$, His, backbone NH), 5.10–5.23 (m, 1H, $C_\alpha H$, Asp), 4.46–4.50 (m, 1H, $C_\alpha H$, His), 3.95–4.37 (m, 2H, $C_\alpha H_2$, Sar), 2.59–3.15 (m, 7H, NCH_3, Sar, $C_\beta H_2$, His/Asp).

Mass calculated: 444.18, found: 444.9 $[M + H]^+$; HPLC retention time: 18.2 min.

TP4: Bz-Glu-Sar-His-NH$_2$ × TFA. ^{1}H-NMR: $\delta = 13.95$ (s, 2H, $NH_{im.prot.}$, His), 7.42–8.91 (m, 9H, CH benzoyl, *C*-term. amide $CONH_2$, $CH_{im.prot.}$, His), 7.27–7.30 (m, 2H, backbone NH), 4.80–4.91 (m, 1H, $C_\alpha H$, Glu), 4.48–4.52 (m, 1H, $C_\alpha H$, His), 3.81–4.11 (m, 2H, $C_\alpha H_2$, Sar), 2.66–3.16 (m, 5H, NCH_3, Sar, $C_\beta H_2$, His), 2.25–2.41 (m, 2H, $C_\gamma H_2$, Glu), 1.86–1.97 (m, 2H, $C_\beta H_2$, Glu).

Mass calculated: 458.19, found: 458.9 $[M + H]^+$; HPLC retention time: 18.9 min.

TP5: Bz-His-Sar-Lys-NH$_2$ × 2 TFA. ^{1}H-NMR: $\delta = 14.17$ (s, 2H, $NH_{im.prot.}$, His), 7.05–8.89 (m, 14H, CH benzoyl, *C*-term. amide $CONH_2$, $CH_{im.prot.}$, His, $\varepsilon NH_{prot.}$, Lys, backbone NH), 5.13–5.25 (m, 1H, $C_\alpha H$, His), 3.84–4.47 (m, 3H, $C_\alpha H$, Lys, $C_\alpha H_2$, Sar), 2.73–3.09 (m, 7H, NCH_3, Sar, $C_\beta H_2$, His, $C_\varepsilon H_2$, Lys), 1.22–1.66 (m, 6H, $C_{\beta/\gamma/\delta}H_2$, Lys).

Mass calculated: 457.24, found: 458.0 $[M + H]^+$; HPLC retention time: 15.9 min.

TP6: Bz-Lys-Sar-Lys-NH$_2$ × 2 TFA. ^{1}H-NMR: $\delta =$ 7.42–8.64 (m, 7H, CH benzoyl, *C*-term. amide $CONH_2$), 7.70 (s, 6H, $\varepsilon NH_{prot.}$, Lys$^{1/3}$), 7.04–7.35 (m, 2H, backbone NH), 4.73–4.87 (m, 1H, $C_\alpha H$, Lys^1), 3.83–4.49 (m, 3H, $C_\alpha H$, Lys^3, $C_\alpha H_2$, Sar), 2.73–2.76 (m, 4H, $C_\varepsilon H_2$, Lys$^{1/3}$), 2.80//3.11 (s, 3H, NCH_3, Sar)*, 1.22–1.71 (m, 12H, $C_{\beta/\gamma/\delta}H_2$, Lys$^{1/3}$).

Mass calculated: 448.28, found: 449.0 $[M + H]^+$; HPLC retention time: 15.7 min.

TP7: Bz-Asp-Sar-Lys-NH$_2$ × TFA. ^{1}H-NMR: $\delta = 12.65$ (s, 1H, $C_\gamma OOH$, Asp), 7.03–8.52 (m, 9H, CH benzoyl, *C*-term. amide $CONH_2$, backbone NH), 7.68 (s, 3H, $\varepsilon NH_{prot.}$, Lys), 4.75–4.83 (m, 1H, $C_\alpha H$, Asp), 3.83–4.18 (m, 3H, $C_\alpha H$, Lys, $C_\alpha H_2$, Sar), 2.73–3.00 (m, 7H, NCH_3, Sar, $C_\beta H_2$, Asp, $C_\varepsilon H_2$, Lys), 1.21–1.72 (m, 6H, $C_{\beta/\gamma/\delta}H_2$, Lys).

Mass calculated: 435.21, found: 435.9 $[M + H]^+$; HPLC retention time: 17.0.

TP8: Bz-Glu-Sar-Lys-NH$_2$ × TFA. ^{1}H-NMR: $\delta = 12.15$ (s, 1H, $C_\delta OOH$, Glu), 7.42–8.61 (m, 7H, CH benzoyl,

C-term. amide $CONH_2$), 7.65 (s, 3H, $\varepsilon NH_{prot.}$, Lys), 7.04–7.39 (m, 2H, backbone NH), 4.84–4.94 (m, 1H, $C_\alpha H$, Glu), 3.79–4.38 (m, 3H, $C_\alpha H$, Lys, $C_\alpha H_2$, Sar), 2.73–3.13 (m, 5H, NCH_3, Sar, $C_\varepsilon H_2$, Lys), 2.28–2.41 (m, 2H, $C_\gamma H_2$, Glu), 1.89–2.02 (m, 2H, $C_\beta H_2$, Glu), 1.22–1.47 (m, 6H, $C_{\beta/\gamma/\delta}H_2$, Lys).

Mass calculated: 449.23, found: 450.0 $[M + H]^+$; HPLC retention time: 17.6 min.

***TP9: Bz-His-Sar-Asp-NH$_2$* × TFA.** ^{1}H-NMR: $\delta = 14.13$ (s, 2H, $NH_{im.prot.}$, His), 12.30 (s, 1H, $C_\gamma OOH$, Asp), 7.40–8.93 (m, 9H, CH benzoyl, *C*-term. amide $CONH_2$, $CH_{im.prot.}$, His), 7.12–7.35 (m, 2H, backbone NH), 5.16–5.26 (m, 1H, $C_\alpha H$, His), 4.42–4.51 (m, 1H, $C_\alpha H$, Asp), 3.79–4.22 (m, 2H, $C_\alpha H_2$, Sar), 2.80–3.11 (m, 5H, NCH_3, Sar, $C_\beta H_2$, His), 2.38–2.71 (m, 2H, $C_\beta H_2$, Asp).

Mass calculated: 444.18, found: 444.7 $[M + H]^+$; HPLC retention time: 17.0 min.

***TP10: Bz-Lys-Sar-Asp-NH$_2$* × TFA.** ^{1}H-NMR: $\delta = 12.33$ (s, 1H, $C_\gamma OOH$, Asp), 7.12–8.65 (m, 12H, CH benzoyl, *C*-term. amide $CONH_2$, backbone NH, $\varepsilon NH_{prot.}$, Lys), 4.75–4.89 (m, 1H, $C_\alpha H$, Lys), 4.44–4.52 (m, 1H, $C_\alpha H$, Asp), 3.75–3.99 (m, 2H, $C_\alpha H_2$, Sar), 2.43–3.09 (m, 7H, NCH_3, Sar, $C_\beta H_2$, Asp, $C_\varepsilon H_2$, Lys), 1.35–1.76 (m, 6H, $C_{\beta/\gamma/\delta}H_2$, Lys).

Mass calculated: 435.21, found: 435.8 $[M + H]^+$; HPLC retention time: 17.6 min.

***TP11: Bz-Asp-Sar-Asp-NH$_2$*.** ^{1}H-NMR: $\delta = 12.27$ (s, 2H, $C_\gamma OOH$, $Asp^{1/3}$), 7.40–8.83 (m, 7H, CH benzoyl, *C*-term. amide $CONH_2$), 7.07–7.31 (m, 2H, backbone NH), 5.12–5.27 (m, 1H, $C_\alpha H$, Asp^1), 4.42–4.54 (m, 1H, $C_\alpha H$, Asp^3), 3.86–4.39 (m, 2H, $C_\alpha H_2$, Sar), 2.77//3.07 (s, 3H, NCH_3, Sar)*, 2.20–2.88 (m, 4H, $C_\beta H_2$, $Asp^{1/3}$).

Mass calculated: 422.14, found: 444.6 $[M + Na]^+$; HPLC retention time: 18.2 min.

***TP12: Bz-Glu-Sar-Asp-NH$_2$*.** ^{1}H-NMR: $\delta = 12.20$ (s, 2H, $C_\gamma OOH$, Asp, $C_\delta OOH$, Glu), 7.44–8.66 (m, 7H, CH benzoyl, *C*-term. amide $CONH_2$), 7.13–7.35 (m, 2H, backbone NH), 4.85–4.99 (m, 1H, $C_\alpha H$, Glu), 4.47–4.56 (m, 1H, $C_\alpha H$, Asp), 3.87–4.41 (m, 2H, $C_\alpha H_2$, Sar), 2.79//3.13 (s, 3H, NCH_3, Sar)*, 2.24–2.72 (m, 4H, $C_\beta H_2$, Asp, $C_\gamma H_2$, Glu), 1.83–2.00 (m, 2H, $C_\beta H_2$, Glu).

Mass calculated: 436.16, found: 458.7 $[M + Na]^+$; HPLC retention time: 18.7 min.

***TP13: Bz-His-Sar-Glu-NH$_2$* × TFA.** ^{1}H-NMR: $\delta = 14.13$ (s, 2H, $NH_{im.prot.}$, His), 12.30 (s, 1H, $C_\delta OOH$, Glu), 7.07–8.94 (m, 11H, CH benzoyl, *C*-term. amide $CONH_2$, $CH_{im.prot.}$, His, backbone NH), 5.12–5.29 (m, 1H, $C_\alpha H$, His), 3.81–4.49 (m, 3H, $C_\alpha H$, Glu, $C_\alpha H_2$, Sar), 2.81–3.18 (m, 5H, NCH_3, Sar, $C_\beta H_2$, His), 2.15–2.24 (m, 2H, $C_\gamma H_2$, Glu), 1.67–1.76 (m, 2H, $C_\beta H_2$, Glu).

Mass calculated: 458.19, found: 458.5 $[M + H]^+$; HPLC retention time: 17.6 min.

***TP14: Bz-Lys-Sar-Glu-NH$_2$* × TFA.** ^{1}H-NMR: $\delta = 12.30$ (s, 1H, $C_\delta OOH$, Glu), 7.07–8.64 (m, 12H, CH benzoyl, *C*-term. amide $CONH_2$, backbone NH, $\varepsilon NH_{prot.}$, Lys), 4.73–4.88 (m, 1H, $C_\alpha H$, Lys), 3.79–4.53 (m, 3H, $C_\alpha H$, Glu, $C_\alpha H_2$, Sar), 2.74–3.11 (m, 5H, NCH_3, Sar, $C_\varepsilon H_2$, Lys), 2.16–2.22 (m, 2H, $C_\gamma H_2$, Glu), 1.22–1.93 (m, 8H, $C_\beta H_2$, Glu, $C_{\beta/\gamma/\delta}H_2$, Lys).

Mass calculated: 449.23, found: 449.8 $[M + H]^+$; HPLC retention time: 18.1 min.

***TP15: Bz-Asp-Sar-Glu-NH$_2$*.** ^{1}H-NMR: $\delta = 12.20$ (s, 2H, $C_\gamma OOH$, Asp, $C_\delta OOH$, Glu), 7.05–8.84 (m, 9H, CH benzoyl, *C*-term. amide $CONH_2$, backbone NH), 5.09–5.26 (m, 1H, $C_\alpha H$, Asp), 4.13–4.31 (m, 1H, $C_\alpha H$, Glu), 3.90–4.06 (m, 2H, $C_\alpha H_2$, Sar), 2.78//3.09 (s, 3H, NCH_3, Sar)*, 2.53–2.87 (m, 2H, $C_\beta H_2$, Asp), 2.03–2.22 (m, 2H, $C_\gamma H_2$, Glu), 1.72–1.76 (m, 2H, $C_\beta H_2$, Glu).

Mass calculated: 436.16, found: 459.2 $[M + Na]^+$; HPLC retention time: 18.9 min.

***TP16: Bz-Glu-Sar-Glu-NH$_2$*.** ^{1}H-NMR: $\delta = 12.10$ (s, 2H, $C_\delta OOH$, $Glu^{1/3}$), 7.41–8.61 (m, 7H, CH benzoyl, *C*-term. amide $CONH_2$), 7.06–7.33 (m, 2H, backbone NH), 4.82–4.92 (m, 1H, $C_\alpha H$, Asp^1), 3.79–4.40 (m, 3H, $C_\alpha H$, Glu^3, $C_\alpha H_2$, Sar), 2.78//3.13 (s, 3H, NCH_3, Sar)*, 2.16–2.41 (m, 4H, $C_\gamma H_2$, $Glu^{1/3}$), 1.67–1.96 (m, 4H, $C_\beta H_2$, $Glu^{1/3}$).

Mass calculated: 450.18, found: 450.9 $[M + H]^+$; HPLC retention time: 19.4 min.

*Different shifts due to *cis*/*trans* conformation, see below.

Ranking of Complex Stabilities

Solutions (1 mM) of the ligand and of transition metal salts (1 mM $Cu(ClO_4)_2$, $Co(ClO_4)_2$, $Ni(ClO_4)_2$, $Zn(ClO_4)_2$ and $Mn(ClO_4)_2$) in deionized water were prepared in Eppendorf PE-tubes. Glass was avoided due to the danger of adsorption of compounds and leaching of alkali ions. The ligand solution (5 µl) was combined with two different metal salt solutions, also 5 µl of each, and incubated at room temperature for 20 min. Doing all possible combinations, this resulted in 10 samples containing (besides the ligand) Cu^{2+}/Co^{2+}, Cu^{2+}/Ni^{2+}, Cu^{2+}/Zn^{2+}, Cu^{2+}/Mn^{2+}, Co^{2+}/Ni^{2+}, Co^{2+}/Zn^{2+},

Co^{2+}/Mn^{2+}, Ni^{2+}/Zn^{2+}, Ni^{2+}/Mn^{2+} or Zn^{2+}/Mn^{2+}. Each sample (1 µl) was mixed with 1 µl of the matrix solution (c = 10 mg/ml 4-nitroaniline in acetonitrile/deionized water = 1 : 1) on a MALDI-plate and measured with 50 shots per spectrum in reflection mode with delayed extraction on a Voyager-DE^{RP} instrument (PerSeptive Biosystems, Framingham, MA, USA) equipped with a nitrogen laser ($\lambda = 337$ nm). In cases where there was no clear predominance of one of the two possible complexes, the complex stabilities were regarded as similar.

Apparent Dissociation Constants for Cu(II)-complexes

All solutions were prepared in 0.01 M phosphate buffer pH 6.4, except for the ligand and $Cu(ClO_4)_2$ stock solutions (1 mM) which were prepared in deionized water. The $Cu(ClO_4)_2$ stock solution was diluted 1 : 100 with buffer to be 10 µM, ligand stock solution was used as it was, providing a sufficient excess of inhibitor over the catalyst. As substrate a 1 mM solution of ascorbic acid in buffer was used, oxygen was found to be at around 0.5 mM in all buffered solutions as measured by an oxygen sensor (Oxi 330, WTW, Weilheim). Ligand and $Cu(ClO_4)_2$ solution were mixed 20 min before adding it to the substrate to allow for complex formation. After adding the complex, the decrease in absorption at 265 nm was recorded on a Lambda 15 UV/VIS-spectrometer (PERKIN-ELMER) for 3 min. A linear fit was performed with the data to obtain first order reaction rates, which were then used in a multidimensional fit to obtain the apparent K_d value. All computation was done using GraFit 3.0 software.

Final concentrations in the cuvettes were as follows: 0.5 µM $Cu(ClO_4)_2$, 20–100 µM ascorbic acid, 10–50 µM ligand and roughly 500 µM of oxygen, that can be considered as being constant over the course of 3 min. Reaction rates were measured at 30 °C.

RESULTS AND DISCUSSION

^{1}H-NMR

Peptides containing imide bonds caused by proline or other *N*-functionalized amino acids are known to be rather stable in the *cis* conformation, causing such peptides to give more than one set of proton signals [17,18]. In our case the difference in chemical shift could be best observed for the *N*-methyl protons of sarcosine, that are adjacent to the *cis*/*trans* peptide bond. In some cases they could be integrated separately, giving a ratio of 70% major and 30% minor conformation, which is in line with the literature given above.

Complex Formation

Conventional methods of measuring complex stabilities such as titration require fairly large amounts of substance. The solid phase synthesis of a larger number of short peptides on a reasonable scale (~250 mg of resin each) only yields around 20 mg highly purified peptide, not enough for repeated analysis of their properties. In our search for alternative methods we came upon mass spectrometry and methods with inherent signal amplification (i.e. catalytic ones). 2 mg of substance is sufficient for ranking of complex stabilities and determining the apparent stability constant of the copper(II)-complex. These methods are described below.

Ranking of Complex Stabilities

In the detection of non covalent interactions, mass spectrometry is employed to an increasing extent [19]. The method needs only a very small amount of substance, however, quantification is rather difficult due to different ionization tendencies of various substances. In our case, ionization ability is mainly a function of the ligand, so the comparison of different metal complexes with one and the same ligand leads to stable and reproducible results. Rather than comparing the complex with the free ligand, which basically means comparing the metal to the proton complex, two metal complexes were compared so that the pH value was not taken into consideration.

Qualitative ranking of complex stabilities for several transition metal cations with TP1–16 was measured by MALDI-TOF mass spectrometry as described earlier by our group [16]. When mixing solutions of the ligand and two different metal salts (p.e. $Cu(ClO_4)_2$ and $Ni(ClO_4)_2$) in an equal molar ratio, the more stable complex is formed predominantly in equilibrium. If this mixture is analysed by MALDI-MS with an appropriate matrix (preferably neutral) the more stable complex can be determined. Usually only one of the two possible complexes is detected alongside the free ligand. Internal control is provided by doing all possible combinations and therefore having plenty of redundancy. If, for example, A is more stable than B, and B is more stable than C, then, of course, A has

also to be more stable than C. In these experiments mixtures of 0.3 mM concentrations of ligands and metal salts in ion-free water were used. The matrix of choice so far is 4-nitroaniline, and a saturated solution in acetonitrile/water 1 : 1 was used. The method works just as well using an ESI-source. Refer to Table 1 for results.

The compounds containing no histidine did not form stable metal complexes under MS-conditions, whereas all histidine containing sequences formed metal complexes. Selectivities for the different metal ions were strongly dependent on the sequence as can be seen by comparing TP3 to TP9. This suggests an involvement of either the *C*-terminal amide group or the *N*-terminal benzoyl group or both in complex formation. Particularly those compounds with a *C*-terminal histidine amide (TP1–4) seem to have a preference for Cu^{2+} ions, while a simple histidine in the sequence is not sufficient for strong Cu^{2+} binding, as is shown by TP9 and TP13, thus pointing to the *C*-terminal amide as the involved end group. However, the presence of at least one histidine seems to be necessary to achieve metal complexation stable enough to be detectable in MS. It is concluded that one histidine, even more a histidine amide, is responsible for the base affinity to the selected metal ions, while the other residue further modifies stability and alters the selectivity of the ligand. This clearly shows that both the side chains as well as at least one of the end groups, probably the amide, are involved in complex formation.

Even though combinatorial approaches give a fast access to a large amount of compounds, the complexation analysis would not yield useful results when used with compound mixtures. It has also been shown by Berkessel and Riedl [20] that the properties of polymer bound substances might not be identical to those of the isolated substances in solution, making an in-depth analysis of isolated compounds necessary. Therefore it was decided to prepare the compounds one by one, especially since the prepared set of 16 pseudotripeptides is rather limited in size.

Apparent Dissociation Constants for Cu(II)-complexes

For the quantification of copper(II) complex stabilities an assay first described by Mottola *et al.* [21] was adapted. Cu^{2+} ions catalyse the oxidation of ascorbic acid following saturation kinetics (Figure 1), which can be measured spectrophotometrically at 265 nm. A ligand forming a copper(II)-complex will act as

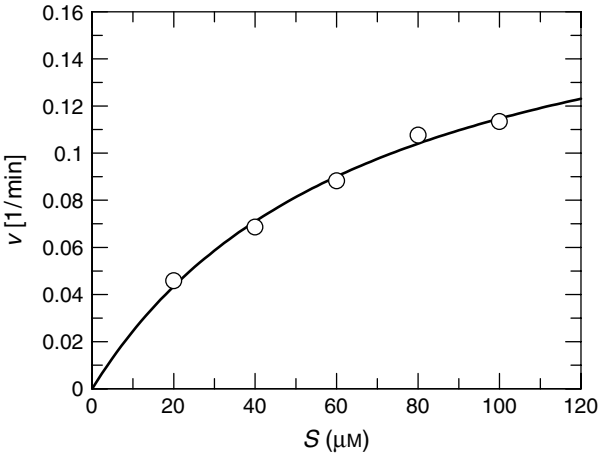

Figure 1 Copper(II) catalysed ascorbate oxidation follows saturation kinetics.

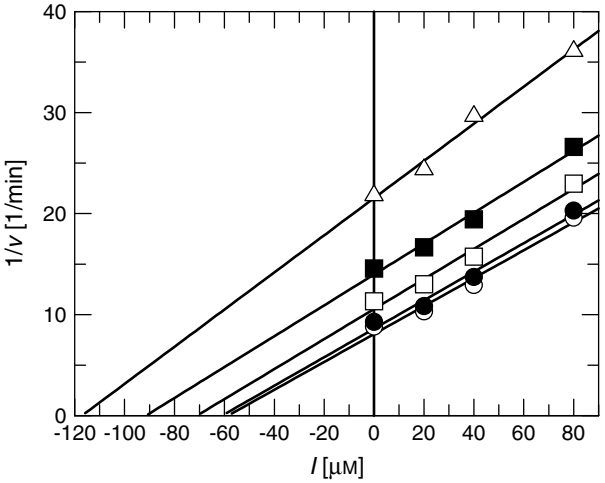

Figure 2 Dixon plot of the inhibition of copper(II) catalysed ascorbate oxidation by TP2. *S* ○, 100 μM; ● 80 μM; □ 60 μM; ■ 40 μM; Δ 20 μM.

an inhibitor, provided the resulting complex is not a catalyst by itself, and therefore the rate of oxidation in the presence of several concentrations of ascorbic acid and ligand can be evaluated by using enzyme kinetics. The Dixon plot shows this kind of inhibition to be uncompetitive (Figure 2), so the apparent K_d, being equivalent to the apparent K_i, can be calculated by directly fitting to Equation 1.

$$v = [v_{max} * S/(1 + I/K_i)]/[S + K_m/(1 + I/K_i)] \quad (1)$$

where initial values for K_m and v_{max} are obtained from a v/S plot in the absence of the ligand. Apparent dissociation constants can be determined

down to 10^{-7} M, which is in line with the limitations described in [21].

The constants are by no means thermodynamic, but conditional constants, suitable only for comparison of compounds analysed under the same conditions. They complement the MS measurements nicely by detecting complexes with stabilities lower than the MS detection level and so allowing the ligands to be fitted on a stability scale of the resulting complexes.

CONCLUSIONS

The complex formation analysis of the pseudotripeptides shows a clear sequence dependence of metal ion selectivity for short peptides, leading to 'complexation modules' for the assembly of larger pseudopeptide ligands. These motifs can also help to understand the preference of metalloenzymes for distinct metal ions. We see them primarily as a prerequisite for the formation of heterodinuclear complexes, that could be models for the heterodinuclear metalloenzymes mentioned above. Such heterodinuclear complexes are difficult to synthesize with peptide ligands. Problems might arise from the fact that the end groups seem to be involved in complex formation, which might lead to unpredictable effects when replacing them by a linker. Furthermore the sequence dependence might also turn out to be extendable to longer peptides, which would make the module approach unfeasible. However, some of the first compounds with the module approach have been synthesized by our group and highly selective heterodinuclear complex formation could be measured. These results will be published separately.

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