Zinc Complexes of Amino Acids and Peptides, 8[◇]

Difunctional Dipeptides Containing Cysteine or Histidine: Solution Behavior and Zinc Complexation

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The dipeptides Ac–Cys–Val–OH (**1a**) and Ac–His–Val–OH (**1b**), H–Gly–Cys–OEt (**2a**) and H–Val–His–OEt (**2b**) as well as Z–Asp–Cys–OH (**3a**) and Z–Asp–His–OH (**3b**) (Z = benzyloxycarbonyl) were prepared, and their zinc complexation was investigated by potentiometric methods. They have in common that in addition to the cysteine thiolate or the histidine imidazole the second amino acid offers one donor function. The complex stabilities are very close to those of the corresponding difunctional derivatives of the single amino acids cysteine or histidine. This indicates the presence of seven- to ten-membered chelate rings.

Histidine and cysteine are the "standard" amino acids for the coordination of metals, specifically zinc, in proteins[2,3], and hence their derivatives and peptides have been the subject of numerous studies of metal complexation equilibria[4]. We have contributed to this by preparative and stability investigations of zinc complexes of histidine[1,5,6] and cysteine[1,7,8] containing peptides as well as of derivatives of the amino acids themselves[9,10].

The exclusive use of the histidine and cysteine donor functions is limited, however, to the so-called structural zinc sites[11] which contain tetrahedrally coordinated noncatalytic zinc for the stabilization of protein loops in enzymes[12], zinc fingers[13] etc.[14]. At catalytic sites there is at least one oxygen donor in the form of the substrate, and many of the popular zinc enzymes like carboxypeptidase and alkaline phosphatase have their zinc in the active center also coordinated by the carboxylate side chains of glutamic or aspartic acid[2,11,15].

It is therefore important to gain knowledge of the zinc complex compositions and stabilities of peptide ligands containing cysteine or histidine in combination with another amino acid offering an additional donor function, specifically carboxylate. This was performed in only a few cases[3,4,16,17], the glutathione (γ-Glu–Cys–Gly) system being investigated most extensively[18]. In order to tackle this problem with peptide ligands which are not available from natural sources (e.g. glutathione), first the synthetic challenge of preparing the highly functional ligands in a pure form has to be met. The quantities necessary for a chemical study further limit the size and the variability of the peptides to be used.

We chose three groups of two dipeptides for an introductory study of this topic. Each group contains the corresponding cysteine and histidine peptides. Peptides **1a** and **b** have the N terminus blocked and contain the amino acid valine at the unprotected carboxyl terminus. Peptides **2a** and **b** bear the additional amino acid at the unprotected amino terminus while the C terminus is blocked. Peptides **3a** and **b** which are potentially tetrafunctional contain cysteine or histidine at the unprotected carboxyl terminus while the second constituent aspartic acid has its N terminus blocked by the Z (benzyloxycarbonyl) group, but bears its free carboxylate function.

Attempts to convert the six peptides into isolable and stoichiometrically defined zinc complexes failed. Their solution behavior was, however, straightforward, and potentiometric titrations yielded complex compositions and stabilities.

Peptide Syntheses

Each of the six peptides chosen contains at least one polyfunctional amino acid constituent. Each peptide synthesis therefore involved the use and removal of several protective groups. The cysteine thiolate function was protected by the trityl (Trt) group or by starting the syntheses with cystine. The histidine imidazole function had to be protected by tritylation only when histidine was the N-terminal amino acid. All C-terminal amino acids were introduced as esters, while the Boc (*tert*-butyloxycarbonyl) function was used as the removable group on the N terminus. Aspartic acid was incorporated together with the Z (benzyloxycarbonyl) function protecting the N terminus and with the γ-carboxylate being protected as the *tert*-butyl (*t*Bu) ester. Peptide coupling was performed either by using the DCC (dicyclohexylcarbodiimide) or the CAIBE (isobutyl chloroformate, i.e.

[◇] Part 7: Ref.[1].

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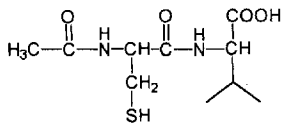
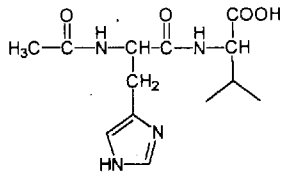
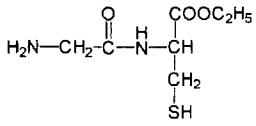


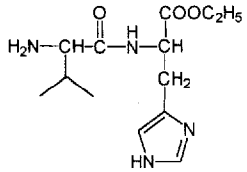
Ac-Cys-Val-OH (**1a**)



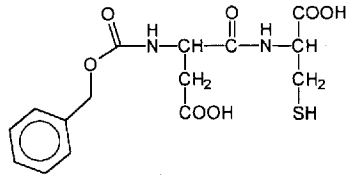
Ac-His-Val-OH (**1b**)



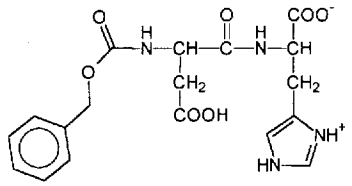
H-Gly-Cys-OEt (**2a**)



H-Val-His-OEt (**2b**)



Z-Asp-Cys-OH (**3a**)



Z-Asp-His-OH (**3b**)

via the mixed anhydrides) method. The fully protected dipeptides thus obtained were:

Ac–Cys(Trt)–Val–OMe **1a′**

Ac–His(Trt)–Val–OMe **1b′**

[Z–Gly–Cys–OEt]$_2$ **2a′**

Boc–Val–His–OEt **2b′**

[Z–Asp(O*t*Bu)–Cys–O*t*Bu]$_2$ **3a′**

Z–Asp(O*t*Bu)–His–OMe **3b′**

They were liberated from their protecting trityl groups, Boc groups and *tert*-butyl ester groups by hydrolysis with trifluoroacetic acid (HTFA), and from their ester groups at the C terminus by alkaline hydrolysis. Cleavage of cystine to cysteine derivatives was performed with dithioerythritol. Reversed-phase HPLC was used for the final purification of the free dipeptides. They were obtained as analytically pure colorless powders in overall yields around 50%. HPLC indicated that they contained varying amounts of HTFA which were determined quantitatively by potentiometric titrations. The identities, constitutions, and purities of the peptides were confirmed by ^{1}H-NMR spectroscopy (see Experimental).

Acid-Base Properties

The peptides **1** and **2** are diprotonic, the peptides **3** are triprotonic acids. Being peptides, they do not contain immediately neighboring ammonium and carboxylate functions, and hence the pK_a values of these functions are to be expected in the normal range of ca. 8 and 3, respectively. Similarly the imidazolium and thiol groups are not affected by strong neighboring effects and should yield pK_a values near 7 and 9, respectively[4,19]. These gross expectations were verified by the potentiometric titrations, whose results are summarized in Table 1.

Table 1. Acid dissociation constants (pK_a) of the dipeptides (standard deviations in parentheses)

peptide	COOH	ImH$^+$	SH	NH_3^+
Ac-Cys-Val-OH (**1a**)	3.52(2)		9.09(3)	
Ac-His-Val-OH (**1b**)	3.18(5)	6.84(3)		
H-Gly-Cys-OEt (**2a**)			8.96(5)	7.95(9)
H-Val-His-OEt (**2b**)		6.06(3)		7.64(5)
Z-Asp-Cys-OH (**3a**)	3.17(8)		9.99(5)	
	4.39(5)			
Z-Asp-His-OH (**3b**)	2.59(8)	7.28(2)		
	3.96(2)			

The inspection of Table 1 reveals some typical trends. One of them is the close similarity of typical pK_a values to those of simple reference compounds: the thiol pK_a of **1a** and **3a** can be compared with that of Ac–Cys–OH (9.62), the imidazolium pK_a of **1b** and **3b** with that of Ac–His–OH (7.11), and the carboxyl pK_a values of **3a** and **3b** with those of Ac–Cys–OH (3.08) and Ac–His–OH (2.94)[10]. Another feature is that comparable pK_a values of the histidine derivatives are always smaller than those of the cysteine derivatives (cf. COOH in **1a/b** or **3a/b** and NH_3^+ in **2a/b**). This is attributed to the fact that the histidine derivative bears one more positive charge than the corresponding cysteine derivative.

Small differences between the pK_a values compiled in Table 1 and those of the reference compounds can be explained by the peculiarities of the peptides. Thus, the thiol pK_a of **1a** is lower than that of Ac–Cys–OH (9.62) because the negatively charged carboxylate function is further away from it in **1a**, and the thiol pK_a of **3a** is higher than that of Ac–Cys–OH because two carboxyl functions have to be deprotonated before the thiol function in **3a**. The same charge difference explains the pK_a difference of about 1 unit for the imidazolium functions in **1b** and **2b** because in **2b** this function is the first to be deprotonated while in **1b** it is the second.

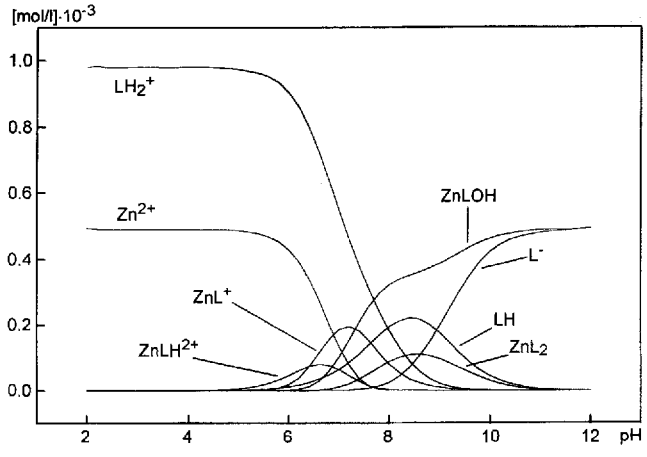
The assignment of the thiol and ammonium pK_a's of **2a** in Table 1 is somewhat ambiguous and was chosen here for the sake of consistency with **1a**, **2b**, and **3a**. It seems unlikely that the ammonium function is deprotonated before the thiol function but it has been discussed in the literature[20]. In our case the common methods to localize sites of deprotonation (e.g. UV-VIS or NMR spectroscopy) did not yield the desired information, and the following discussion does not depend on the specific assignment of the two pK_a's.

Zinc Complexation

Each of the six dipeptide ligands was subjected to three potentiometric titrations in the presence of zinc nitrate, the L-to-Zn concentration ratios being roughly 4:1, 2:1, and 1:1. Complexation was immediately obvious from the typical pH depression. In some cases, specifically **1b** and **3b**, the titrations suffered from precipitations at pH $\geq$ 8. This indicates the formation of oligomeric species, and it impeded the quantitative assessment of high pH species. In the case of **1b** and **3b** it can be related to the low stabilities of the zinc complexes allowing the formation of insoluble basic zinc salts.

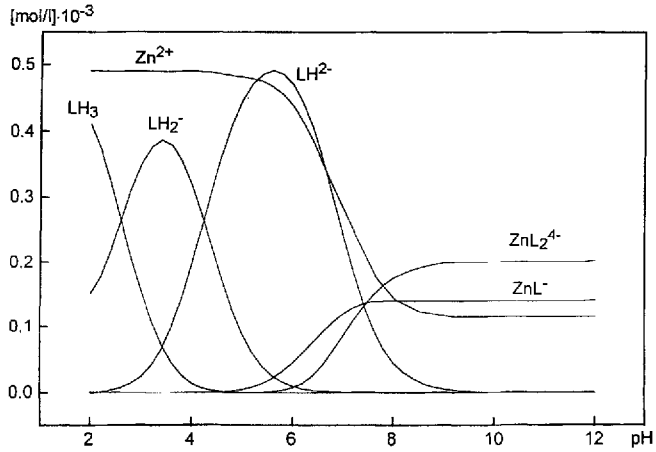
The evaluation of the titration curves produced the species distributions in solution. Figures 1 and 2 show two typical examples. They not only reveal that various complexes and the free ligands coexist in neutral and basic solution (which may explain why pure compounds could not be isolated) but also visualize the charge effects: peptide H-Gly-Cys−OEt is cationic (LH_2^+) in the fully protonated state and monoanionic as the zinc-bound ligand, peptide Z−Asp−Cys−OH is a neutral triprotonic acid (LH_3) in the free state and accordingly trianionic in the ligated form. This has a strong influence on the complex formation (ZnL_2 vs. ZnL_2^{4-} etc.) and on the acid-base properties of the complexes themselves (e.g. **2a** and **b** allow the formation of ZnOH species in solution but the other peptides do not).

Figure 1. Species distribution in a solution of H−Gly−Cys−OEt (**2a**) at a peptide:Zn ratio of 2:1



The species distributions correspond to the overall stability constants of the complexes which are expressed as logβ in Table 2 according to the usual definitions (β_{110} for ZnL, β_{120} for ZnL_2 etc.[1]). The table shows that only the ZnL complexes could be detected for all peptides. The ZnL_2 complexes were found in four cases, and typically in the two cases where they are missing (for histidine containing **1b** and **3b**) the stabilities of the ZnL complexes are already quite low. As a rule (exception **2a**), the gain in complex stability in going from ZnL to ZnL_2 is roughly the same as that for the formation of ZnL which may also be anticipated for the unobserved ZnL_2 complexes of **1b** and **3b**. For this reason the pertinent information can be obtained from a discussion of the ZnL complex stabilities.

Figure 2. Species distribution in a solution of Z−Asp−Cys−OH (**3a**) at a peptide:Zn ratio of 1:1



Normally one would expect that the gain in complex stability in going from ZnL to ZnL_2 to be smaller than the stability constant of ZnL, e.g. $\log K_1 > \log K_2$. That **1a**, **1b**, and **3a** do not follow this rule may be related to a change in coordination geometry. The latter ist not uncommon in zinc chemistry, e.g. for complexes of penicillamine[4] or cyclo-HisHis[21]. Alternatively the omission of Zn−OH species from the calculations could produce unrealisitically high $\log\beta_2$ values, but this was checked and dismissed in the present study. Furthermore the possible existence of dinuclear zinc complexes in solution was eliminated by means of the calculations.

Table 2. Stability constants (log β) for the zinc-dipeptide complexes (standard deviation in parentheses)

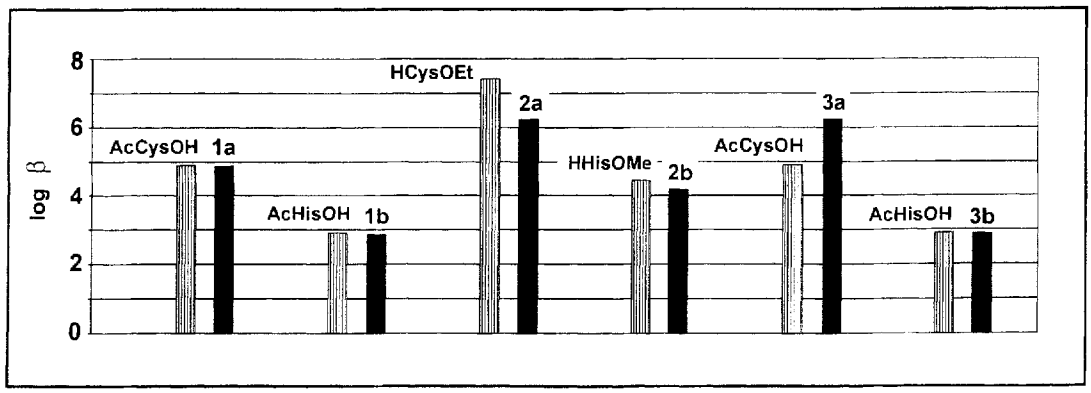
peptide		ZnL	ZnL_2
Ac-Cys-Val-OH	(**1a**)	4.89(20)	10.55(10)
Ac-His-Val-OH	(**1b**)	2.89(6)	
H-Gly-Cys-OEt	(**2a**)[a]	6.25(14)	8.91(15)
H-Val-His-OEt	(**2b**)[b]	4.20(8)	8.19(8)
Z-Asp-Cys-OH	(**3a**)	6.25(20)	12.70(20)
Z-Asp-His-OH	(**3b**)	2.90(3)	

[a] $\log\beta$ (ZnLH) = 12.93(10), $\log\beta$ (ZnLOH) = −0.93(10). − [b] $\log\beta$ (ZnL_2OH) = 0.98(18).

The donor sets offered by the peptide ligands are S/O (**1a**, **3a**), N/O (**1b**, **3b**), S/N (**2a**), and N/N (**2b**). None of these is arranged such that the favored five-membered chelate ring can be formed. The ZnL complex stabilities are therefore expected to be lower than those of ideal ligands like mercaptoacetic acid ($\log\beta$ = 7.80[22]), glycine ($\log\beta$ = 5.19[23]), H−Cys−OEt ($\log\beta$ = 7.42[10]) or histamine ($\log\beta$ = 5.62[24]). This is observed, as is the general phenomenon that the sulfur-containing ligands (S/O or S/N) provide ZnL complex stabilties about two orders of magntiude higher than those of the corresponding nitrogen-containing ligands (N/O or N/N).

On the other hand, it is notable that the ZnL complexes of the dipeptides are not generally less stable than those

Figure 3. Complex stabilities (logβ for ZnL) of simple amino acid donors (light bars) in comparison to those of the dipeptides (dark bars)



of the corresponding monoamino acid derivatives. Figure 3 illustrates this graphically for **1**–**3** in comparison with the data for the appropriate N- or C-protected cysteine and histitidine derivatives taken from ref.[10]. The figure shows that in four of the six cases the two complexes are practically identical in stability. Only the highly favored H–Cys–OEt complex is more stable than that of H–Gly–Cys–OEt. In contrast, Z–Asp–Cys–OH forms a considerably more stable complex than its simple analog Ac–Cys–OH.

For the first four cases (peptides **1** and **2**) the peptides and their mono-amino acid models both offer two donor atoms. In the latter two cases (peptides **3**) the peptides offer three and the models two such atoms. The chelate ring size of the models is 5 for H–Cys–OEt, 6 for Ac–Cys–OH and H–His–OMe, and 7 for Ac–His–OH. The figure shows that only the most suitable chelate ring size of H–Cys–OEt can offer an additional stabilization of the model complex. This leads to the conclusion that the bidentate peptide ligands **1a**, **b**, and **2a**, **b** form chelate complexes of good stability despite their unfavorable chelate ring sizes of 8 (**2a**), 9 (**1a** and **2b**), and 10 (**1b**). The amount of stability gained by chelation can also be judged by making reference to the ZnL complex stabilities of the monodentate amino acid ligands Ac–Cys–NH_2 and Ac–His–NH_2 for which logβ values of 4.77 and 1.96 were found[25].

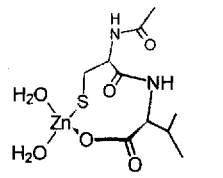
The situation for peptides **3** is more complicated because the additional carboxylate function of their aspartic acid constituent is not present in the model ligands Ac–Cys–OH and Ac–His–OH. The stabilizing effect of this function is obvious from the increased stability of the ZnL complex of **3a**. In contrast, the ZnL complex of **3b** has gained no higher stability than that of Ac–His–OH. Because the cysteine and histidine constituents of peptides **3** are located at the unprotected C terminus the principal chelate ring sizes in their complexes (6 for **3a** and 7 for **3b**) are identical to those of the model complexes. The additional carboxylate coordination would create a 10-membered chelate ring for **3a** and a 11-membered chelate ring for **3b**. The observed result is an enhancement of the zinc-cysteine complex stability while the zinc-histidine complex stability remains low and obviously cannot be improved either by formation of a ZnL_2 complex.

Conclusions

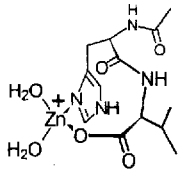
The di- or trifunctional peptides **1**–**3** form zinc complexes of remarkable stability. The main reason for this seems to be their ability to act as chelating ligands. The peculiarities of peptide conformations enable their chelate rings to be relatively strain-free even for the resulting ring sizes of 8 to 10. Formulas **1A**–**3C** represent the proposed constitutions of the ZnL complexes of peptides **1a**–**3c**. The formulas were constructed by allowing only the "good" donors, e.g. thiolate, amine, and imidazole, to coordinate to zinc and taking into consideration that coordination by carbonyl oxygen is practically unknown in zinc enzymes. In the absence of any direct structural information these constitutions cannot be anything but proposals. It should be mentioned, however, that we have proved the existence of 9-, 12-, and 15-membered chelate rings in zinc complexes of bis(cysteinyl) peptides[26].

With respect to the zinc coordination in proteins peptides **2a** and **b** are not relevant because they contain the unprotected N-terminal NH_2 function. While the same might be said about the other four peptides which contain unprotected C termini, the combination of their C-terminal carboxyl function with the second or third function of the peptide creates as bonding situation (thiolate + carboxylate or imidazole + carboxalate) which is natural. The high stability of this bonding situation, specifically for **1a** and **3a**, reflects its occurrence in zinc-containing enzymes[2,4,11].

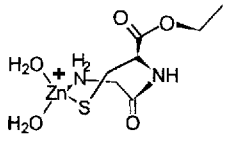
Our observations underline statements on biocoordination that we have made for simpler amino acid and peptide complexes of zinc before[5–8]: histidine and histidine-containing peptides are amazingly weak ligands in contrast to their high importance for metal coordination in proteins. The ZnL complex stabilities of **1b**, **2b** and **3b** show that even the chelate effect or N/O and N/N donor combinations do not improve this situation much. Furthermore, non-natural bonding situations like the amine/thiolate chelation by H–Gly–Cys–OEt or H–Cys–OEt can be significantly



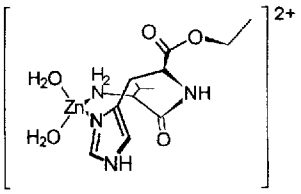
1A



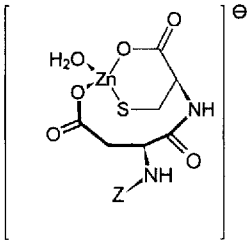
1B



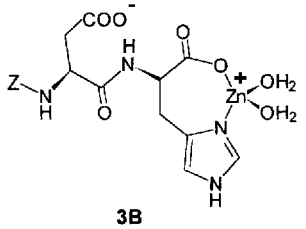
2A



2B



3A



3B

better than the natural ones provided by peptide side chains. Both statements point to preorganization as the dominating factor determining binding stabilities and specificities of metal ions in proteins.

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Experimental

The general working and measuring methods are described in ref.[9], the HPLC procedures are described in ref.[6]. All starting materials were obtained commercially. The following abbreviations are used: Z: benzyloxycarbonyl, Boc: *tert*-butyloxycarbonyl, DCC: dicyclohexylcarbodiimide, HOBT: hydroxybenzotriazole, NMM: *N*-methylmorpholine, NEM: *N*-ethylmorpholine, HTFA: trifluoroacetic acid, CAIBE: chloroformic acid isobutyl ester, DTE: dithioerythritol.

The raw dipeptides were worked up according to the following procedure: the ethyl acetate extract was washed 3 times with 50 ml of water each, 3 times with 50 ml of acetate buffer solution (pH 4.0), 3 times with 50 ml of water, 3 times with 50 ml of 4% $NaHCO_3$ solution and 4 to 5 times with 50 ml of water (until the filtrate had neutral pH). Then the ethyl acetate phase was dried with Na_2SO_4 overnight and evaporated to dryness in vacuo.

N^{α}-Acetyl-L-cysteinyl-L-valine (Ac–Cys–Val–OH, **1a**): 1.93 g (4.8 mmol) of N^{α}-acetyl-(*S*-trityl)cysteine and 1.00 g (4.8 mmol) of valine *tert*-butyl ester hydrochloride were dissolved separately each in 40 ml of THF. The solutions were cooled to −12 °C and 0.55 ml (0.51 g, 5.0 mmol) of NMM was added to each solution. After stirring for 15 min the solution containing *N*-acetyl-(*S*-trityl)cysteine was treated with 0.65 ml (0.68 g, 5.0 mmol) of CAIBE, and after an additional 15 min both solutions were combined. Stirring was continued for 24 h at room temp., the solvent removed in vacuo and the residue extracted with 110 ml of a mixture of ethyl acetate and water (10:1). The workup (see above) yielded 2.57 g (96%) of Ac–Cys(Trt)–Val–O*t*Bu as a colorless solid. – 2.57 g (4.6 mmol) of the solid was dissolved in 20 ml of ethane thiol and the solution treated with 15 ml of HTFA. After standing for 3 h at room temp., all volatile solvents were removed in vacuo and the remaining yellow solid was extracted with 50 ml of water. The residue was dissolved in 50 ml of diethyl ether and the solution extracted with four 40-ml portions of water. The combined aqueous layers were evaporated to dryness in vacuo. Preparative HPLC (water/acetonitrile, 85:15, flow rate 6 ml/min) of the crude product and freeze drying of the eluent yielded 0.63 g (49%) of **1a** as a colorless powder, m.p. 260 °C. – IR (KBr, cm^{-1}): $\tilde{\nu}$ = 3315 s (NH), 3084 m, 2965 s, 2927 m, 2874 w (CH, CH_3), 2564 m (SH), 1722 s, 1651 s, 1541 s (CO), 1467 w, 1422 m, 1376 m, 1298 w, 1265 w, 1209 s, 1147 m (CN), 1037 w, 1019 w, 988 w, 800 w, 722 w. – ^{1}H NMR (D_2O): δ = 0.97 (d, *J* = 6.9 Hz, 3H, CH_3-Val), 0.98 (d, *J* = 6.8 Hz, 3H, CH_3-Val), 2.07 (s, 3H, Ac), 2.22 (dsept, *J* = 6.0 Hz, *J* = 6.8 Hz, 1H, CHβ-Val), 2.87 (dd, *J* = 6.9 Hz, *J* = 14.1 Hz, 1H, CHβ-Cys), 2.96 (dd, *J* = 6.1 Hz, *J* = 14.1 Hz, 1H, CHβ-Cys), 4.31 (d, *J* = 5.9 Hz, 1H, CHα-Val), 4.55 (dd, *J* = 6.9 Hz, *J* = 6.1 Hz, 1H, CHα-Cys). – $C_{10}H_{20}N_2O_5S$ (280.3): calcd. C 42.81, H 7.14, N 9.99; found C 42.93, H 6.58, N 9.51.

N^{α}-Acetyl-L-histidyl-L-valine (Ac–His–Val–OH, **1b**): 1.50 g (3.4 mmol) of N^{α}-acetyl-(N^{im}-trityl)-histidine and 1.04 g (3.4 mmol) of valine methyl ester tosylate were dissolved in 13 ml of degassed dimethylformamide. At −5 °C the mixture was treated with 0.50 ml (0.35 g, 3.5 mmol) of triethylamine, 0.46 g (3.4 mmol) of HOBT and 0.78 g (3.8 mmol) of DCC. After stirring for 24 h at room temp. the mixture was filtered, the solvent removed in vacuo and the residue extracted with two 50-ml portions of ethyl acetate. The workup (see above) yielded 1.43 g (76%) of Ac–His(Trt)–Val–OMe as a colorless oil. – 300 mg (0.56 mmol) of the oil was treated at 0 °C with 1 ml of HTFA. After stirring for 6 h at room temp. all volatile components were removed in vacuo and the remaining yellow oil was extracted five times with 10 ml of diethyl ether each. The remaining solid was dried in vacuo to yield 230 mg (96%) of Ac–His–Val–OMe · HTFA as a colorless powder, m.p. 156 °C. – 230 mg (0.54 mmol) of this powder was dissolved in 5 ml of methanol, the solution treated with 5 ml of 0.1 M NaOH at 0 °C, and stirred for 24 h at room temp. It was then neutralized with 0.1 M HCl and evaporated to dryness in vacuo. The residue was dissolved in 3 ml of water. Preparative HPLC (water/acetonitrile, 80:20, flow rate 6 ml/min) and freeze drying of the eluate yielded 0.19 g (73%) of **1b** · 1.7 HTFA as a colorless powder, m.p. 209 °C. – IR (KBr, cm^{-1}): $\tilde{\nu}$ = 3298 m, 3149 s, 2899 s (br., NH, OH), 1727 m, 1656 vs, 1641 vs, 1544 m (CO), 1206 m (CF_3). – ^{1}H NMR (D_2O): δ = 0.80 (d, *J* = 3.0 Hz, 3H, CH_3-Val), 0.83 (d, *J* = 3.0 Hz, 3H, CH_3-Val), 1.91 (s, 3H, Ac), 2.03–2.11 (m, 1H, CHβ-Val), 3.07–3.13 (m, 2H, $C\beta H_2$-His), 4.17 (d, *J* = 5.5 Hz, 1H, CHα-Val), 4.58 (m, 1H, CHα-His), 7.22 (s, 1H, H^5-His), 8.53 (d, *J* = 1.2 Hz, 1H, H^2-His). – $C_{13}H_{20}N_4O_4$ · 1.7 $F_3C_2O_2H$ (296.1 + 194.0): calcd. C 39.97, H 4.42, N 11.30; found C 39.49, H 4.64, N 12.09.

Glycyl-L-cysteine Ethyl Ester (H–Gly–Cys–OEt, **2a**): 3.82 g (20.0 mmol) of *tert*-butyloxycarbonyl glycine was dissolved in 100 ml of THF, the solution cooled to −12 °C and treated with 2.20 ml (2.02 g, 20.0 mmol) of NMM, followed by 2.60 ml (2.72 g, 20.0 mmol) of CAIBE. After 5 min this solution was combined with 50 ml of a cooled (−12 °C) dimethylformamide solution containing 6.41 g (10.0 mmol) of cystine diethyl ester ditosylate and 2.20 ml (2.02 g, 20.0 mmol) of NMM. Stirring was continued for 16 h at

room temp., the mixture filtered and the volume of the solvent reduced in vacuo to 25 ml. 80 ml of water was added and the solution extracted with three 30-ml portions of ethyl acetate. The workup (see above) yielded 5.80 g (95%) of [Boc–Gly–Cys–OEt]$_2$ as a colorless oil. – 5.80 g (9.50 mmol) of the oil was dissolved in 50 ml of HTFA at room temp. After 1 h the solvent was removed in vacuo and the oily residue triturated with five 20-ml portions of diethyl ether. Drying of the solution in vacuo yielded 5.11 g (84%) of [H–Gly–Cys–OEt]$_2$ · HTFA as a colorless oil. – 0.92 g (1.43 mmol) of this oil and 0.42 g (2.72 mmol) of DTE were dissolved in 30 ml of degassed water. After adjustment of the pH to 9.0 with 0.2 M NaOH and stirring for 20 h the solution was acidified to pH 2 with an excess of HTFA, the volatile components were removed in vacuo and the remaining solid was washed with three 10-ml portions of diethyl ether. Preparative HPLC (water/acetonitrile, 80:20, flow 6 ml/min) and freeze drying of the eluate yielded 50 mg (6%) of **2a** · HTFA as a colorless hygroscopic powder, m.p. 82°C. – IR (KBr, cm^{-1}): $\tilde{\nu}$ = 3130 s (NH), 3070 m, 2989 m, 2921 m, 2552 w (SH), 1646 s (CO), 1550 s (CO), 1430 w, 1372 m, 1342 m, 1301 m, 1204 s, 1183 m (CF$_3$), 1134 m, 1024 m, 915 w, 835 w, 799 w, 723 w. – ^{1}H NMR (CD$_3$CN): δ = 1.27 (t, J = 7.1 Hz, 3H, CH$_3$-ethyl), 2.74–3.12 (m, 2H, CβH$_2$-Cys), 3.80 (d, J = 17.3 Hz, 1H, CHα-Gly), 3.95 (d, J = 17.3 Hz, 1H, CHα-Gly), 4.16 (q, J = 7.1 Hz, 2H, CH$_2$-ethyl), 4.24–4.28 (m, 1H, CHα-Cys), 6.65 (s, 1H, NH-Cys). – $C_7H_{14}N_2O_3 \cdot C_2F_3O_2H$ (174.2 + 114.0): calcd. C 37.50, H 5.24, N 9.72; found C 36.97, H 5.13, N 9.56.

L-Valyl-L-histidine Ethyl Ester (H–Val–His–OEt, **2b**): 6.15 g (28.3 mmol) of *tert*-butyloxycarbonylvaline was dissolved in 100 ml of THF, the solution cooled to –12°C and treated with 4.21 ml (3.87 g, 28.3 mmol) of NEM followed by 3.12 ml (3.26 g, 28.3 mmol) of CAIBE. After 5 min this solution was combined with 100 ml of a cooled (–12°C) THF solution containing 5.19 g (28.3 mmol) of histidine ethyl ester dihydrochloride and 8.42 ml (6.52 g, 56.6 mmol) of NEM. Stirring was continued for 24 h at room temp., the solvent removed in vacuo and the residue dissolved in 100 ml of ethyl acetate. The workup (see above) yielded 8.86 g (82%) of Boc–Val–His–OEt as a colorless solid. – At 0°C 8.86 g (23.2 mmol) of the solid was dissolved in 10 ml of HTFA and the mixture stirred for 35 min at room temp. The volatile components were removed in vacuo and the remaining solid was washed with three 20-ml portions of diethyl ether to yield 11.7 g (23.0 mmol, 99%) of H–Val–His–OEt · 2 HTFA as a colorless oil. Preparative HPLC (water/acetonitrile, 95:5, flow 6 ml/min) of 2 g of this raw product and freeze drying of the eluent yielded 1.40 g (63%) of **2b** · 2.5 HTFA as a colorless powder, m.p. 74°C. – IR (KBr, cm^{-1}): $\tilde{\nu}$ = 3400 s (br, CONH), 3142 s (NH_3^+), 2960 s (CH$_3$), 2654 s (br, =NH$^+$–), 1742 m, 1671 st (CO), 1566 s (CO), 1546 s, 1498 s, 1466 m (CH$_2$), 1378 s, 1203 vs (CF$_3$), 1137 vs (CO), 837 m, 798 m, 722 m (CH$_2$). – ^{1}H NMR (D$_2$O): δ = 1.01, 1.06 (d, d, J = 3.0 Hz, 6H, CH$_3$-Val), 1.22 (t, J = 6.9 Hz, 3H, CH$_3$-ethyl), 2.23–2.27 (m, 1H, CHβ-Val), 3.18–3.44 (m, 2H, CβH$_2$-His), 3.79 (d, J = 6.00 Hz, 1H, CHα-Val), 4.21 (q, J = 6.9 Hz, 2H, CH$_2$-ethyl), 4.80–4.84 (m, 1H, CHα-His), 7.34 (s, 1H, H^5-His), 8.65 (d, 1H, H^2-His). – $C_{13}H_{22}N_4O_3 \cdot 2.5\ C_2F_3O_2H$ (282.2 + 285.0): calcd. C 38.10, H 4.35, N 9.87; found C 38.56, H 4.73, N 10.45.

N$^\alpha$-Benzyloxycarbonyl-L-aspartyl-L-cysteine (Z–Asp–Cys–OH, **3a**): 1.03 g (3.2 mmol) of N$^\alpha$-benzyloxycarbonyl-(*O-tert*-butyl)aspartic acid and 0.67 g (1.58 mmol) of cystine di-*tert*-butyl ester dihydrochloride were each dissolved separately in 20 ml of THF. The solutions were cooled to –12°C and treated with 0.70 ml (0.65 g, 3.2 mmol) of NMM each. After stirring for 15 min the solution containing N$^\alpha$-benzyloxycarbonyl-(*O-tert*-butyl)aspartic acid was treated with 0.42 ml (0.44 g, 3.2 mmol) of CAIBE, and after additional 15 min both solutions were combined. Stirring was continued for 24 h at room temp., the solvent removed in vacuo and the residue extracted with 100 ml of ethyl acetate. The workup (see above) yielded 1.43 g (96%) of [Z–Asp(O*t*bu)–Cys–O*t*Bu]$_2$ as a colorless oil. – 1.31 g (1.39 mmol) of the oil was dissolved in 10 ml of HTFA at room temp. After 1 h the solvent was removed in vacuo and the oily residue triturated with five 10-ml portions of diethyl ether. Drying in vacuo yielded 0.93 g (91%) of [Z–Asp–Cys–OH]$_2$ as a colorless, extremely hygroscopic solid. – 0.93 g (1.26 mmol) of this solid and 0.33 g (2.14 mmol) of DTE were dissolved in 30 ml of degassed water. After adjustment of the pH to 8.5 with 0.2 M NaOH and stirring for 3 h the solution was acidified to pH 2 with an excess of HTFA, and the volatile components were removed in vacuo. Washing of the remaining solid with three 10-ml portions of diethyl ether yielded 1.06 g (71%) of pure **3a** as a colorless solid, m.p. 170°C. – IR (KBr, cm^{-1}): $\tilde{\nu}$ = 3338 s (NH), 3050 m, 2950 m, 2581 m (SH), 1721 sh (CO), 1685 m (aryl), 1639 s (CO), 1524 s (CO), 1428 w, 1400 m, 1327 w, 1293 w, 1271 m, 1208 m, 1137 w, 1055 m, 845 w, 804 m, 779 w, 740 w, 727 m, 697 m (aryl), 670 w. – ^{1}H NMR (D$_2$O): δ = 2.68–3.00 (m, 4H, CβH$_2$-Cys, CHβ-Asp), 4.42–4.48 (m, 1H, CHα-Cys), 4.58 (dd, J = 8.0 Hz, J = 5.6 Hz, 1H, CHα-Asp), 5.16–5.22 (m, 2H, CH$_2$-Z), 7.36–7.46 (m, 5H, aryl-H-Z). – $C_{15}H_{18}N_2O_7S$ (370.1): calcd. C 48.64, H 4.90, N 7.56; found C 48.59, H 5.24, N 7.79.

N$^\alpha$-Benzyloxycarbonyl-L-aspartyl-L-histidine (Z–Asp–His–OH, **3b**): 2.50 g (7.7 mmol) of N$^\alpha$-benzyloxycarbonyl-(*O-tert*-butyl)aspartic acid and 1.30 g (7.7 mmol) of histidine methyl ester were dissolved in 50 ml of acetonitrile. At 0°C the mixture was treated with 1.62 g (7.9 mmol) of DCC. After stirring for 16 h at room temp. half ot the solvent was removed in vacuo, the remaining mixture filtered and evaporated to dryness. The residue was dissolved in 5 ml of HTFA, which was removed in vacuo and added again three times. After 1 h the solution was evaporated to dryness and the remaining residue triturated with 20 ml of diethyl ether to yield 2.60 g (59%) of Z–Asp–His–OMe · HTFA as a colorless solid, m.p. 132°C. – 1.49 g (2.60 mmol) of the colorless solid was dissolved in dioxane/water (80:20), and 2.76 ml (5.35 mmol) of 2 M NaOH was added to the solution. After stirring for 30 min the pH was adjusted to 2 with 2 M HCl, the solution evaporated to dryness in vacuo and the residue dissolved in 5 ml of water. Preparative HPLC (water/acetonitrile, 70:30, flow 6 ml/min) and freeze drying of the eluate yielded 1.06 g (71%) of **3b** · 1.5 HTFA as a colorless solid, m.p. 144°C. – IR (KBr, cm^{-1}): $\tilde{\nu}$ = 2959 m, 1762 vs, 1625 m (CO), 1598 m (COO asym.), 1514 m (CO), 1459 m, 1433 m, 1290 s (COO sym.), 1079 m, 1063 m, 1000 m, 957 m, 830 m. – ^{1}H NMR (D$_2$O): δ = 2.71–2.94 (m, 2H, CβH$_2$-Asp), 3.08–3.41 (m, 2H, CβH$_2$-His), 4.51–4.56 (m, 1H, CαH-Asp), 4.75–4.79 (m, 1H, CαH-His), 5.16 (s, 2H, CH$_2$-benzyl), 7.25 (s, 1H, H^5-His), 7.40–7.46 (m, 5H, H-phenyl), 8.52 (d, J = 1.2 Hz, 1H, H^2-His).

Table 3. Starting concentrations (· 10^{-3} M) of the reagents for the potentiometric titrations

peptide	c(peptide)	c(acid)	c(zinc salt)
1a	0.800	3.060	0.266/0.444/1.070
1b	1.100	1.700	0.289/0.577/1.160
2a	1.000	1.350	0.251/0.502/1.004
2b	0.982	1.380	0.251/0.502/1.004
3a	0.550	0.430	0.236/0.447/0.894
3b	0.914	0.520	0.224/0.447/0.894

– $C_{18}H_{20}N_4O_7 \cdot 1.5\ C_2F_3O_2H$ (404.4 + 171.0): calcd. C 44.01, H 3.71, N 10.40; found C 43.83, H 3.77, N 9.74.

Potentiometric Titrations: The apparatus used, the experimental details and the calibration techniques are described in ref.[10]. The data acquisition and all computations were performed by using Zuberbühler's TITFIT program[27]. The common anion in all solutions was nitrate [KNO_3, $Zn(NO_3)_2$, HNO_3]. Before starting the titrations an exact amount of acid (HNO_3) was added, the titration base was 0.20 M NaOH. The starting concentrations of peptides, acid, and zinc salts are given in Table 3.

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