71. Metal Complexes with Macrocyclic Ligands

Part XLIV[1])

Kinetics of the Cu^{2+} Incorporation into a Macrocyclic Ligand Conjugated to Proteins: Model Studies for the 'Post-Labeling' Technique

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The kinetics of the Cu^{2+} complexation by macrocycles **1** (4-[(1,4,8,11-tetraazacyclotetradec-1-yl)methyl]-benzoic acid) and **2** (*N*-propyl-4-[(1,4,8,11-tetraazacyclotetradec-1-yl)methyl]benzamide) as well as by macrocycle **1** conjugated to bovine serum albumin (bsa) and to ribonuclease A (rnase) were studied by stopped flow techniques. For **1** and **2**, the kinetics were followed in the mM range monitoring the d-d* absorption band of the Cu^{2+} complex. From the pH dependence of k_{obs}, the rate law is $v = [Cu^{2+}](k_{LH}[LH] + k_{LH_2}[LH_2])$, where k_{LH} and k_{LH_2} are the bimolecular rate constants for Cu^{2+} with the diprotonated (LH_2) and monoprotonated (LH_1) form of the ligand, respectively. The values are $k_{LH_2} = 1.7(1)$ $M^{-1}s^{-1}$ and $k_{LH} = 2.3(1) \cdot 10^5$ $M^{-1}s^{-1}$ for **1**, and $k_{LH_2} = 0.28(9)$ $M^{-1}s^{-1}$ and $k_{LH} = 2.0(1) \cdot 10^5$ $M^{-1}s^{-1}$ for **2**. The kinetics of the Cu^{2+} incorporation into **1**, **2** and **1** conjugated to bsa and rnase, *i.e*, **3** and **4**, respectively, were also followed using nitroso-R salt as a metal indicator in the μM range, *i.e.*, under conditions typical for the 'post-labeling' technique to give radiolabeled monoclonal antibodies. In these cases, the reaction takes place between the 1:1 complex of Cu^{2+} with nitroso-R-salt and the macrocycle. At pH 6.5, the rates are very similar to each other indicating that the complexation properties of the macrocycle attached to a protein are not very different from those of the free ligand under comparable conditions.

Introduction. – Radiolabeled monoclonal antibodies have a great potential for diagnosis and therapy in tumor-affected patients [2] [3]. Generally, the antibody is modified by attaching to it a chelating group, which then can bind a radioactive metal ion. Among the radionuclides, γ-emitters such as ^{111}In, ^{99m}Tc, ^{67}Ga, ^{64}Cu, *etc.* can be used for diagnostic purposes, whereas β-emitters, such as ^{90}Y, ^{67}Cu, ^{111}Ag, ^{188}Re, *etc.* are ideal for therapy [3]. To label modified antibodies, two methods have been used so far: the 'pre-labeling' technique, which allows a selective incorporation of the radionuclide before the ligand is attached to the monoclonal antibody, and the 'post-labeling' technique, in which the complexing unit is first conjugated to the antibody, and the radioactive metal ion is thereafter incorporated.

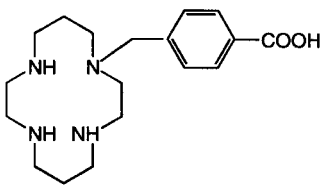
Although the 'post-labeling' method is easier to apply as a kit for medical routine purposes, it suffers from several problems. First the metal complexation has to be done under nondenaturing conditions at neutral pH, at which some metal ions precipitate as hydroxides; secondly, the kinetics of incorporation must be fast, an important aspect, since one deals with second-order reaction rates in the μM range; and third, there is the possibility that the metal ion not only binds to the complexing unit conjugated to the

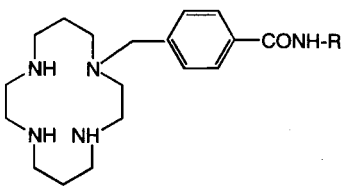
[1]) Part XLIII: [1].

antibody, but also unspecifically at coordinating groups of the protein and is then released once in the body fluid.

It has previously been shown, that 4-[(1,4,8,11-tetraazacyclotetradec-1-yl)methyl]-benzoic acid (**1**, cpta) can be conjugated to antibodies such as AB35 directed against carcino embryonal antigen (CEA) without significant loss of immuno reactivity, and that the conjugate can be labeled with $^{67}Cu^{2+}$ in acetate buffer with a full occupancy of the macrocycle in less than 20 min [4].

In continuation of such studies, we present here a new investigation on the structure of the Cu^{2+} complex with **1** and on the rate of Cu^{2+} complexation with the free macrocyclic ligands **1** and **2**, and with **1**, attached to bovine serum albumin (bsa) or ribonuclease A (rnase), **3** and **4**, respectively, as models for antibodies under the typical conditions for the 'post-labeling' technique.





2 R = $(CH_2)_2CH_3$
3 R = bsa
4 R = rnase

Experimental. – *General.* All reagents and solvents, obtained from commercial sources, were used without purification. IR Sprectra: as KBr pellets (1–3%) on a *Perkin-Elmer 1600 FTIR.* ^{1}H- and ^{13}C-NMR Spectra: at 25° on a *Varian Gemini 300* spectrometer using 5 to 30 mg substance dissolved in 0.5 ml of D_2O or $CDCl_3$. Reported chemical shifts are relative either to TMS ($CDCl_3$) or sodium 3-(trimethylsilyl)propane-sulfonic acid (D_2O). Elemental analysis were performed at the analytical laboratory of *Ciba-Geigy AG*, Basel.

X-Ray Diffraction Measurements. The structure analysis of the Cu^{2+} complex with **1** was carried out with MoK_α X-rays on an *Enraf Nonius CAD 4* diffractometer equipped with a MoK_α fine-focus sealed tube and a graphite monochromator. Cell parameters were obtained by least-squares procedure on 25 independent strong reflections. The crystal system is $P\bar{1}$: $a = 6.816$ Å; $b = 8.690$ Å; $c = 21.233$ Å; $\alpha = 79.40°$; $\beta = 81.21°$; $\gamma = 77.10°$. The temp. of the measurement was 173 °K, the absorption coefficient 11.727 cm^{-1} and the extinction parameter 41.913. No significant variation in the intensities of three standard reflections monitored every 120 min was observed. 7269 Reflections were observed and 5993 were used for the refinement. The data set was corrected as usual and calculated by the direct methods [5]. Beside the H-atoms all other atoms were refined anisotropically with CRYSTALS [6] by taking the atom form factors from the *International Tables for X-Ray Crystallography* [7]. H-Atoms on N and O were kept with restraints on a distance of 1 Å, and their position was refined. $R = 3.33\%$, $wR = 3.65\%$; $w = [1 - (\Delta(F)/6\sigma F)^2]^2$.

Syntheses. 4-[(1,4,8,11-Tetraazacyclotetradec-1-yl)methyl]benzoic acid tetrahydrochloride (**1** · 4 HCl; cpta · 4 HCl) and *{4-[(1,4,8,11-tetraazacyclotetradec-1-yl)methyl]benzoato}copper(II) perchlorate* were synthesized as described in [8].

N-*Propyl-4-[(1,4,8,11-tetraazacyclotetradec-1-yl)methyl]benzamide Tetrahydrochloride* (**2**). Under dry N_2, a soln. of dicyclohexylcarbodiimide (4.27 g, 20 mmol) in MeCN (80 ml) was added dropwise to a mixture of *N*-propylamine hydrochloride (1.91 g, 20 mmol) and {4-[(1,4,8,11-tetraazacyclotetradec-1-yl)methyl]benzoato}-copper(II) perchlorate (2.62 g, 5 mmol) in MeCN (150 ml) under reflux. After 2 h, the mixture was cooled to r.t.

and kept overnight. H_2O was added and the residue filtered. After evaporation of MeCN, the soln. was continuously extracted during 8 h with CH_2Cl_2. Then, NaOH (1.67 g) was added to the soln., and the extraction was continued for further 20 h. To the H_2O phase, NaCN (1.67 g) and MeOH (30 ml) were added, and the soln. was heated under reflux until the blue color disappeared. MeOH was evaporated, and the soln. was extracted with CH_2Cl_2. After evaporating the org. solvent, the residual oil was taken up with MeOH (25 ml). The product was precipitated by addition of 36% HCl (5 ml). IR (KBr): 3415 (NH), 2964 (CH), 2780 (NH^+), 1637 cm^{-1} (C=O). ^{1}H-NMR: 0.91 (*t*, Me); 1.60 (*m*, MeCH_2); 2.16 (*quint.*, 2 $CH_2CH_2CH_2$); 3.2–3.7 (*m*, 9 CH_2N); 4.40 (*s*, $C_6H_4CH_2$); 7.61 (*d*, 2 arom. H); 7.81 (*d*, 2 arom. H). ^{13}C-NMR: 17.74 (Me); 25.50, 26.00 ($CH_2CH_2CH_2$); 28.97 ($MeCH_2$); 44.76, 44.90, 48.38, 48.52, 48.66, 48.90, 51.99, 55.09, 55.98, 65.52 (CH_2N); 135.12, 138.22, 139.77, 142.81 (arom. C); 177.2 (CO). Anal. calc. for $C_{21}H_{37}N_5O \cdot 4.1\ HCl \cdot 2\ H_2O \cdot 0.4$ MeOH (567.78): C 44.76, H 8.20, Cl 25.37, N 12.20, O 9.47; found: C 44.60, H 8.18, Cl 25.35, N 12.17, O 9.17.

{4-[(1,4,8,11-Tetraazacyclotetradec-1-yl)methyl]benzoic acid}copper(II) dichloride was obtained by dissolving **1** · 4 HCl (500 mg, 1.03 mmol) and $CuCl_2 \cdot 2H_2O$ (175 mg, 1.03 mmol) in H_2O (20 ml) and neutralizing the soln. with 1M NaOH (4.1 ml). The solvent was evaporated to *ca.* 5 ml. By addition of EtOH, NaCl was precipitated and filtered off. To crystallize the product, H_2O was slowly evaporated. Yield: 350 mg (71%). VIS (H_2O): λ_{max} 514 (133). IR (KBr): 3344 (COOH), 3144 (NH), 2872 (CH), 1712 (C=O). Anal. calc. for $C_{18}H_{30}Cl_2CuN_4O_2 \cdot 1.1\ H_2O$ (487.54): C 44.24, H 6.64, Cl 14.51, Cu 13.00, N 11.46, O 10.15; found: C 44.02, H 6.63, Cl 14.78, Cu 12.85, N 11.49, O 10.23.

Protein Modifications. Modified beef serum albumin (bsa) was obtained by dissolving **1** · 4 HCl (10 mg) in 0.1M NaOH (0.4 ml) and 0.01M phosphate buffer (pH 6.5, 0.5 ml), to which *N*-[3-(dimethylamino)propyl]-*N'*-ethylcarbodiimide hydrochloride (20 mg) and *N*-hydroxysuccinimide (5 mg) were added. After 15 min, the mixture was given dropwise to bsa (*Merck 12018, fraction V*, 100 mg) in 0.01M phosphate buffer (pH 6.5, 100 ml). After staying overnight at r.t., the modified protein **3** was isolated by gel filtration. The measured ratio of macrocycle to protein was 2.7.

Modified ribonuclease A (rnase) was obtained by dissolving **1** · 4 HCl (45 mg) in 0.1M NaOH (1.8 ml) and 0.01M phosphate buffer (pH 6.5, 0.5 ml), to which *N*-[3-(dimethylamino)propyl]-*N'*-ethylcarbodiimide hydrochloride (90 mg) and *N*-hydroxysuccinimide (22 mg) were added. After 15 min, the mixture was reacted with rnase (*Fluka 83831*, 100 mg) in 0.01M phosphate buffer (pH 6.5, 25 ml). After staying overnight at r.t., the modified protein **4** was isolated by gel filtration. The measured ratio of macrocycle to protein was 2.3.

The modified proteins were purified by gel filtration over a *Sephadex G25* column using a *LKB Bromma 2232 Microperpex* peristaltic pump and a *LKB Bromma 2238 UVCORD SII* as UV detector at 254 nm.

Determination of the Macrocyle-to-Protein Ratio. The modified proteins were incubated with an excess of $CuSO_4$. The excess and the unspecifically bound Cu^{2+} were complexed by edta and separated from the protein by gel filtration over *Sephadex G25*. The concentration of the protein was measured spectrophotometrically at 280 nm ($\varepsilon = 63000\ M^{-1}cm^{-1}$ for bsa and $\varepsilon = 10000\ M^{-1}cm^{-1}$ for rnase), and the concentration of Cu^{2+} was measured by atomic absorption spectrometry (*Pye Unicam SP 2900*). It was assumed that the Cu^{2+} concentration is equal to macrocycle concentration.

Kinetics. For the stopped-flow measurements at 25°, a *Perkin-Elmer Lambda 2* spectrophotometer equipped with a hand-driven mixing device or a *Durrum D110* stopped-flow instrument on line with a PC were used. The evaluation of the kinetics was performed using a pseudo first-order rate law.

The rate of Cu^{2+} complex formation with **1** and **2** was measured following the absorbance at 514 nm. The pH was varied between 3.86 and 5.76 using 0.1M lutidine-3-sulfonic acid as buffer ($I = 0.5$ with KNO_3), the Cu^{2+} and the ligand concentration were $1 \cdot 10^{-3}$ M and $1 \cdot 10^{-4}$ M, resp.

In the same pH range, the formation of the Cu^{2+} complex with **1** was also measured using the metal indicator nitroso-R salt (2-hydroxy-1-nitrosonaphthalene-3,6-disulfonic acid disodium salt) at 484 nm. Typical concentrations were: $[Cu^{2+}] = 1 \cdot 10^{-4}$ M, [nitroso-R salt] $= 2 \cdot 10^{-4}$ M and [ligand] $= 1 \cdot 10^{-5}$ M. In addition, a nitroso-R-salt dependence at pH 4.96 was also measured under the same conditions. The concentration of the nitroso-R salt was varied between $2 \cdot 10^{-4}$ M and $6 \cdot 10^{-4}$ M.

The rate of formation of the Cu^{2+} complexes with **1**–**4** was also measured at pH 6.5 in 0.01M phosphate buffer ($I = 0.1$ with KNO_3) using nitroso-R salt of different concentrations at 484 nm. The Cu^{2+} concentration was $0.8 \cdot 10^{-4}$ M, the concentration of the nitroso-R salt varied between $1.6 \cdot 10^{-4}$ M and $4 \cdot 10^{-4}$ M, and the ligand or protein soln. were prepared, so that the macrocycle concentration was $8 \cdot 10^{-4}$ M.

Results and Discussion. – Since **1** has been shown to be easy to prepare [8] [9], and it has good properties to bind Cu^{2+} and to be conjugated to monoclonal antibodies

through amide bond formation [4], it was interesting to study it as Cu^{2+} complex and as amide derivative **2**. The structure of the Cu^{2+} complex with **1** was solved by X-ray diffraction measurements (*Fig. 1*). Since the crystals were grown from acidic solution, the protonated species $[CuLH]^{2+}$ was formed. The proton is clearly sitting at the O-atom of the carboxy group and forms a H-bond to a H_2O molecule $H_2O(3)$.

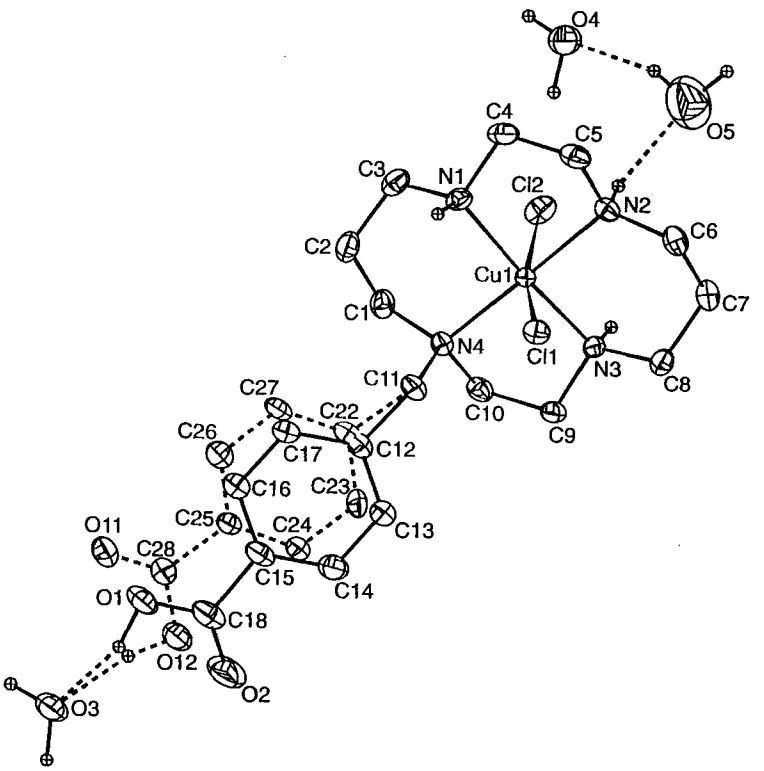


Fig. 1. *ORTEP Plot of the Cu^{2+} complex with* **1**, *showing the H-bonds to three H_2O molecules and the disorder of the side chain*

In $[CuLH]^{2+}$, the macrocycle is in the *trans-III*-configuration, which is the thermodynamically most stable arrangement for a 14-membered tetraazamacrocycle [10]. The four N-atoms form a nearly perfect plane with small deviation of $\pm$ 0.021 Å, and the Cu^{2+} ion is only slightly displaced from this plane by 0.097 Å, so that with the macrocycle a square-planar arrangement results. In addition, two chlorides Cl(1) and Cl(2) are axially coordinated at 2.81 and 2.93 Å, respectively. This gives an elongated octahedral coordination geometry and is due, from the tendency of Cu^{2+}, to give *Jahn-Teller*-distorted structures. Cu–N Bonds are in the normal range; interesting is, however, that the Cu–N(4) bond, to the N-atom, which is substituted by the side chain, is somewhat longer than the other ones (*Table 1*). There is some disorder in the side chain, which takes up to positions with an occupancy of 0.33 and 0.67 (*Fig. 1*).

Table 1. *Selected Bond Distances* [Å] *and Angles* [°] *in* $[CuLH]^{2+}$ (L = **1**)

Cu(1)–Cl(1)	2.8093(3)	Cl(1)–Cu(1)–Cl(2)	169.60(1)	N(1)–Cu(1)–N(3)	173.02(4)
Cu(1)–Cl(2)	2.9319(4)	Cl(1)–Cu(1)–N(1)	91.30(3)	N(2)–Cu(1)–N(3)	92.32(5)
Cu(1)–N(1)	2.008(1)	Cl(2)–Cu(1)–N(1)	91.31(3)	Cl(1)–Cu(1)–N(4)	97.33(3)
Cu(1)–N(2)	2.040(1)	Cl(1)–Cu(1)–N(2)	86.92(3)	Cl(2)–Cu(1)–N(4)	92.48(3)
Cu(1)–N(3)	2.002(1)	Cl(2)–Cu(1)–N(2)	83.24(3)	N(1)–Cu(1)–N(4)	94.95(5)
Cu(1)–N(4)	2.103(1)	N(1)–Cu(1)–N(2)	85.64(5)	N(2)–Cu(1)–N(4)	175.69(4)
		Cl(1)–Cu(1)–N(3)	95.25(3)	N(3)–Cu(1)–N(4)	86.60(4)
		Cl(2)–Cu(1)–N(3)	81.82(3)		

Ligand **2** was synthesized by activation of **1** through the *N*-succinimide ester which, by addition of *N*-propylamine, was converted into the amide. To characterize the new ligand, the log K_H values were determined by potentiometry (*Table 2*).

Table 2. *Protonation Constants of* **1**, **2**, *and Cyclam at 25° and* I = *0.5*M (KNO_3)

	Cyclam [12]	**1**	**2**
log K_{H1}	11.83	11.43(5)	11.16(3)
log K_{H2}	10.76	9.29(1)	9.13(1)
log K_{H3}		3.87(1)	

As for many tetraazamacrocycles we find two high values, which can be attributed to the protonation of two N-atoms *trans* to each other [11]. The third log K_H value of **1** probably represents the protonation of the carboxy group, since it is absent for **2**.

The modifications of the proteins bsa and rnase were performed by activation of the carboxy group of **1** with a water-soluble carbodiimide to give the *N*-hydroxysuccinimide ester, which was then added to the protein solution. By this procedure, it was possible to attach 2.7 to 11 macrocycles to bsa and 0.9 to 2.3 macrocycles to rnase. The number of macrocycles attached to the proteins was determined by saturating the protein with Cu^{2+} so that all macrocycles became complexed. The excess of Cu^{2+} and the Cu^{2+} bound at unspecific sites were then removed by adding edta and purifying the protein over *Sephadex G50*. The Cu^{2+} content of the protein fraction was determined by atomic absorption spectrometry, and the protein concentration was calculated from its absorptivity at 280 nm. In this way, the macrocycle-to-protein ratio could be evaluated.

Before the complexation of Cu^{2+} with the macrocycle attached to the proteins was studied, the reactivity of the free ligands **1** and **2** was measured for comparison. This was performed either by following the absorptivity change at 514 nm, which corresponds to the d-d* band of the Cu^{2+} chromophore, or at 484 nm using nitroso-R salt as a metal indicator. Following the d-d* band, we found that the observed pseudo-first-order rate constant k_{obs} (10-fold excess of Cu^{2+} over ligand) depends on [Cu^{2+}] and the pH (*Figs. 2* and *3*).

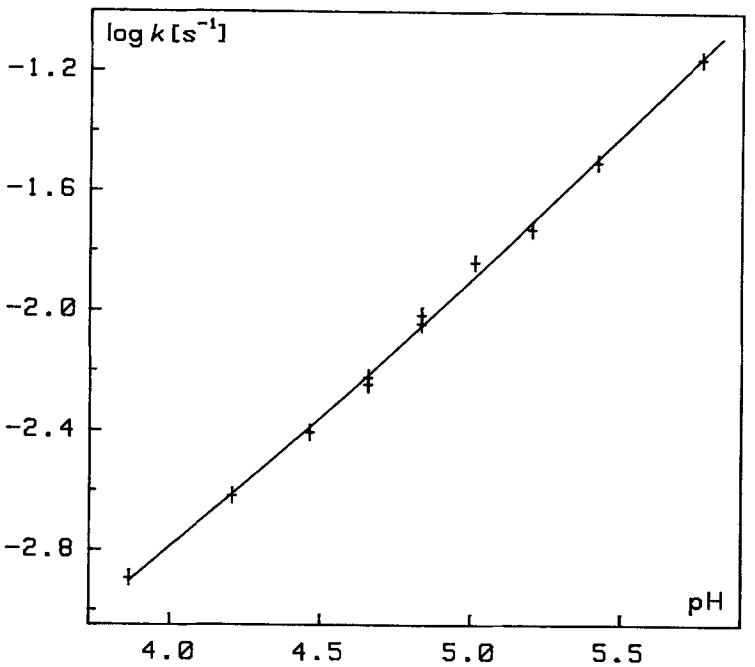


Fig. 2. *pH Dependence of* k_{obs} *for the complexation of* Cu^{2+} *with* **1**. + : Experimental points, – : calculated curve.

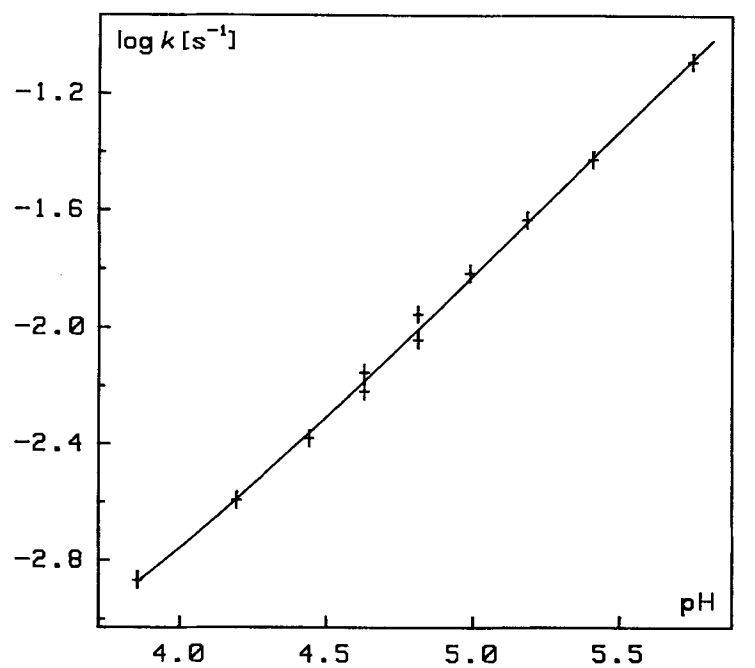


Fig. 3. *pH Dependence of* k_{obs} *for the complexation of* Cu^{2+} *with* **2**. + : Experimental points, – : calculated curve.

The mathematical equations describing the system (*Scheme 1*) are *Eqns. 1–3*, where a_H is the proton activity,

$$K_{H2} = [LH_2]/([LH] \cdot a_H) \quad (1)$$

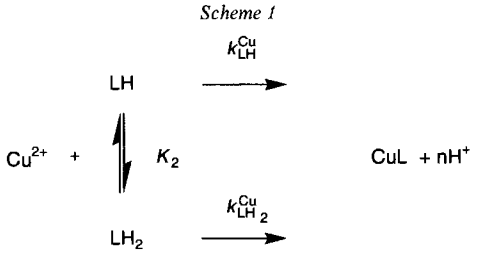
$$C_L = [LH_2] + [LH] \quad (2)$$

$$v = (k_{LH}[LH] + k_{LH_2}[LH_2])\,[Cu^{2+}] = k_{obs}\, C_L \cdot [Cu^{2+}] \quad (3)$$

This gives for k_{obs} *Eqn. 4*:

$$k_{obs} = (k_{LH}^{Cu}\, K_{H2} + k_{LH_2}^{Cu}\, a_H)/(K_{H2} + a_H) \quad (4)$$

Scheme 1



With *Eqn. 4*, the experimental data were fitted using k_{LH}^{Cu} and $k_{LH_2}^{Cu}$ as variable parameters and keeping K_{H2} constant at the values given in *Table 2*. The quality of the fitting can be seen from *Figs. 1* and *2*, and the results are given in *Table 3*.

Table 3. *Bimolecular Rate Constants for the Complexation of Cu^{2+} and [CuX] with Cyclam,* **1**, *and* **2** *at 25° and* I = *0.5*M

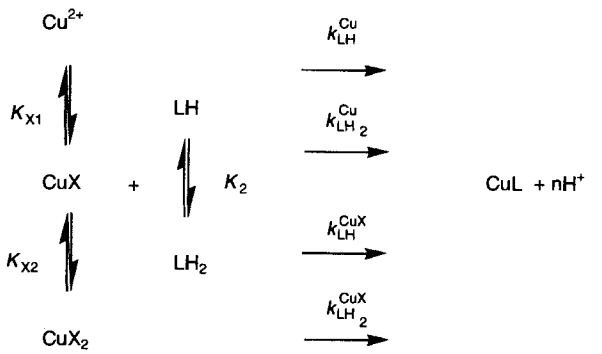
	Cyclam	**1**	**2**
k_{LH}^{Cu} [M^{-1}s^{-1}]	$1.8 \cdot 10^6$ [12]	$2.3(1) \cdot 10^5$	$2.0(1) \cdot 10^5$
$k_{LH_2}^{Cu}$ [M^{-1}s^{-1}]	0.39 [12]	1.7(1)	0.28(9)
k_{LH}^{CuX} [M^{-1}s^{-1}]		$1.7(1) \cdot 10^6$	
$k_{LH_2}^{CuX}$ [M^{-1}s^{-1}]		22(1)	

Compared to the unsubstituted macrocycle 1,4,8,11-tetraazacyclotetradecane (cyclam), the values of k_{LH}^{Cu} for **1** and **2** are smaller by a factor 10, indicating that *N*-substitution lowers somewhat the reactivity probably for steric reasons. The values of $k_{LH_2}^{Cu}$ are distinctly different for **1** and **2**, macrocycle **1** reacting about six times faster than **2**, which is more similar to cyclam. Since $k_{LH_2}^{Cu}$ is strongly dependent on electrostatic factors [12], our result shows that the negative charge of the carboxy group in **1**, although relatively far from the macrocyclic unit, is promoting the complexation with Cu^{2+} compared to cyclam and **2**, which do not have this group.

The spectrophotometric method used for these experiments, by which the absorbance of the d*-d* band of the Cu^{2+} chromophore is measured, does not allow to go below the mM concentration range, since the molar absorptivity of the Cu^{2+} macrocycles is only *ca.* 100–150 $M^{-1}cm^{-1}$. To overcome this problem and to do experiments in the μM concentration range, which is typical for the 'post-labelling' technique, we had to use a metal indicator with a high molar absorptivity but a not too high complexation constant. After several attempts, nitroso-R salt (2-hydroxy-1-nitroso-3,6-dimethylnaphthalene-3,6-disulfonic acid disodium salt) seemed a good candidate for such measurements.

Through addition of nitroso-R salt, the reaction scheme becomes more complex, since besides the solvated metal ion there are also the complexes with nitroso-R salt ([CuX] and [CuX_2]), and these also have their own reactivity in the complexation reaction (*Scheme 2*).

Scheme 2



The kinetics of Cu^{2+} incorporation into **1** and **2** were measured as a function of the metal concentration, nitroso-R-salt concentration, and the pH. To simultaneously fit the pseudo-first-order constants k_{obs} from the pH dependence and the nitroso-R-salt dependence at pH 4.96, we had to assume that the free Cu^{2+} as well as 1:1 complex [CuX] are reactive with LH and LH_2, whereas the 1:2 complex [CuX_2] is not reactive at all. This model gives the following *Eqns. 5–11*.

$$K_{HX} = [HX]/([X] \cdot a_H) \tag{5}$$

$$K_{X1} = [CuX]/([Cu^{2+}] \cdot [X]) \tag{6}$$

$$K_{X2} = [CuX_2]/([Cu^{2+}] \cdot [X]^2) \tag{7}$$

$$K_{H2} = [LH_2]/([LH] \cdot a_H) \tag{8}$$

$$C_L = [LH_2] + [LH] \tag{9}$$

$$C_{Cu} = [Cu^{2+}] + [CuX] + [CuX_2] \tag{10}$$

$$C_X = [HX] + [X] + [CuH] + 2[CuX_2] \tag{11}$$

Combining *Eqns. 5–10*, and *11* gives a third-order equation in [X], which was solved using the algorithm given in [13]. Once [X] is known the $[Cu^{2+}]$ concentration can be calculated from *Eqn. 10*.

For the rate law, we have *Eqn. 12*, from which k_{obs} can be determined (*Eqn. 13*).

$$v = (k_{LH}^{Cu}[LH] + k_{LH_2}^{Cu}[LH_2])[Cu^{2+}] + (k_{LH}^{CuX}[LH] + k_{LH_2}^{CuX}[LH_2])\,[CuX] \qquad (12)$$

$$k_{obs} = \{k_{LH}^{Cu}K_{H2} + k_{LH_2}^{Cu}a_H + (k_{LH}^{Cu}K_{H2} + k_{LH_2}^{CuX}a_H)K_{X1}[X]\}[Cu^{2+}]/((K_{H2} + a_H)C_{Cu}) \qquad (13)$$

Using *Eqn. 13*, the pH and the nitroso-R-salt dependencies of **1** were fitted together in a batch procedure (*Fig. 4*). Thereby k_{LH}^{Cu} and $k_{LH_2}^{Cu}$ were taken from *Table 2*, and K_{HX} and K_{X1} are from [14]. K_{X2}, k_{LH}^{CuX}, and $k_{LH_2}^{CuX}$ were used as variable parameters to fit the experimental values. The results listed in *Table 2* show that $\log K_{X2} = 15.32$ ([14]: 14.51) gives the best fit. The rate constants of [CuX] with LH and LH_2 are distinctly higher than those of the free Cu^{2+} ion. The effect of ligands attached to Cu^{2+} on the reactivity of the metal incorporation into macrocycles has been previously observed and discussed [15]. The higher reactivity, especially towards LH_2, is due to the smaller electrostatic interaction between the Cu^{2+} complex and the protonated ligand. In this respect, our results with nitroso-R salt nicely fit into this picture, since [CuX] has a lower positive charge than Cu^{2+}.

In a last series of experiments, the Cu^{2+} incorporation in **1**–**4** under *quasi*-physiological conditions at pH 6.5 in 0.01M phosphate buffer was followed using nitroso-R salt as metal indicator. To fit the nitroso-R-salt dependences for **1**–**4**, we used the simplified *Scheme 3* in which Cu^{2+} is present as free ion, as 1:1 complex [CuX], and 1:2 complex $[CuX_2]$. The rate constant k^{CuX} is a conditional constant for pH 6.5 and has to be used, since the protonation degree of the ligands, especially when covalently attached to the proteins, is not known.

The same *Eqns. 5–7, 10*, and *11* valid for *Scheme 2* can be used to calculate the concentrations of [X] and $[Cu^{2+}]$. Testing different models, we found that only [CuX] is reactive. Thus, the rate law is given by *Eqn. 14*.

$$v = k^{CuX}\,[L]\,[CuX] = k_{obs}[L]C_{Cu} \qquad (14)$$

which gives for k_{obs} *Eqn. 15*

$$k_{obs} = k^{CuX} \cdot K_{X1}[X]\,[Cu^{2+}]/C_{Cu} \qquad (15)$$

The values so obtained are given in *Table 4* and the fitting is shown in *Fig. 5*.

The results of *Table 4* allow to compare the reactivity of the macrocyclic unit either as free ligand or conjugated to a protein. Under the conditions chosen for the incorporation (pH 6.5), it is not possible to have free Cu^{2+} ion, since it hydrolyzes and precipitates as hydroxide. However, with the addition of a ligand such as nitroso-R salt one can keep Cu^{2+} in solution as a complex, and it is the [CuX] species which reacts with the macrocycle. Since the protonation constants of the macrocycle attached to the protein are not known, and we are working at only one pH value, only the conditional rate constants k^{CuX} can be determined. The reactivities of **1**, **2**, and **1** conjugated to bsa and

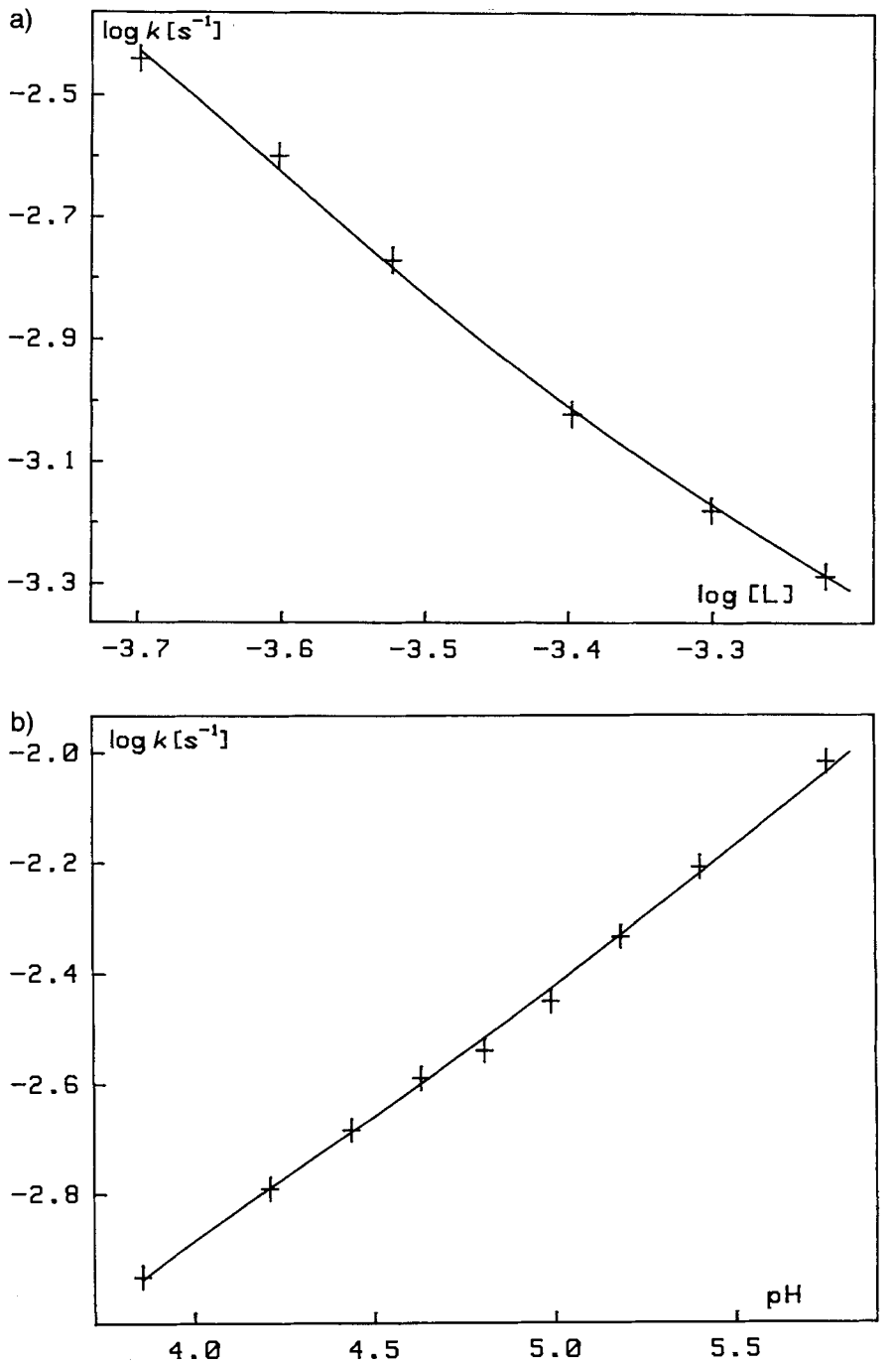


Fig. 4. *Nitroso-R salt* (a) *and pH dependences* (b) *for the complexation of* Cu^{2+} *with* **1** *using the metal indicator.* + : Experimental points, – : calculated curve.

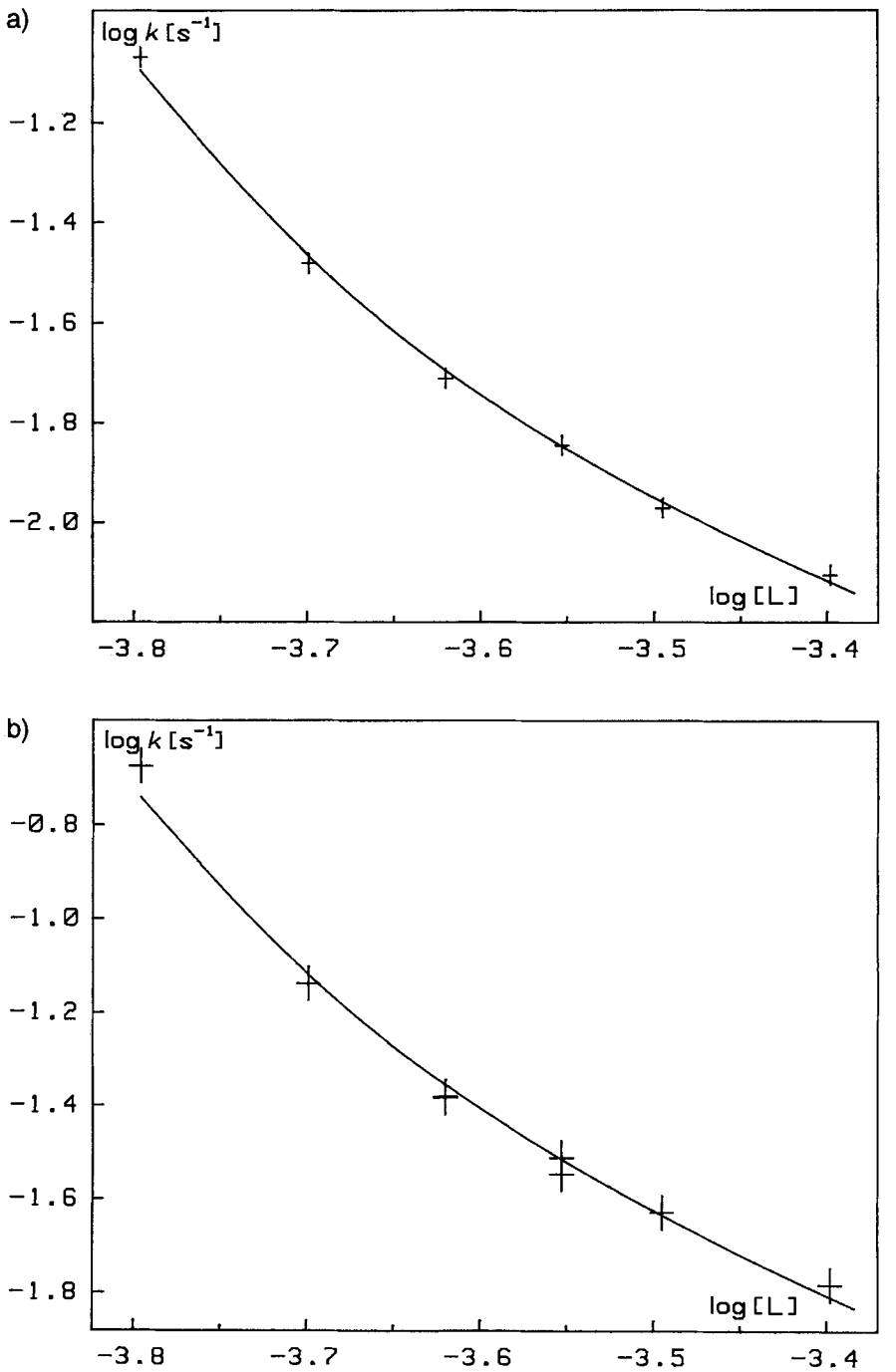


Fig. 5. *Nitroso-R-salt dependencies for the complexation of* Cu^{2+} *with* **1** (a), **2** (b), **3** (c), *and* **4** (d) *at pH 6.5, 25°, and* I = *0.1*M. + : Experimental points, – : calculated curve.

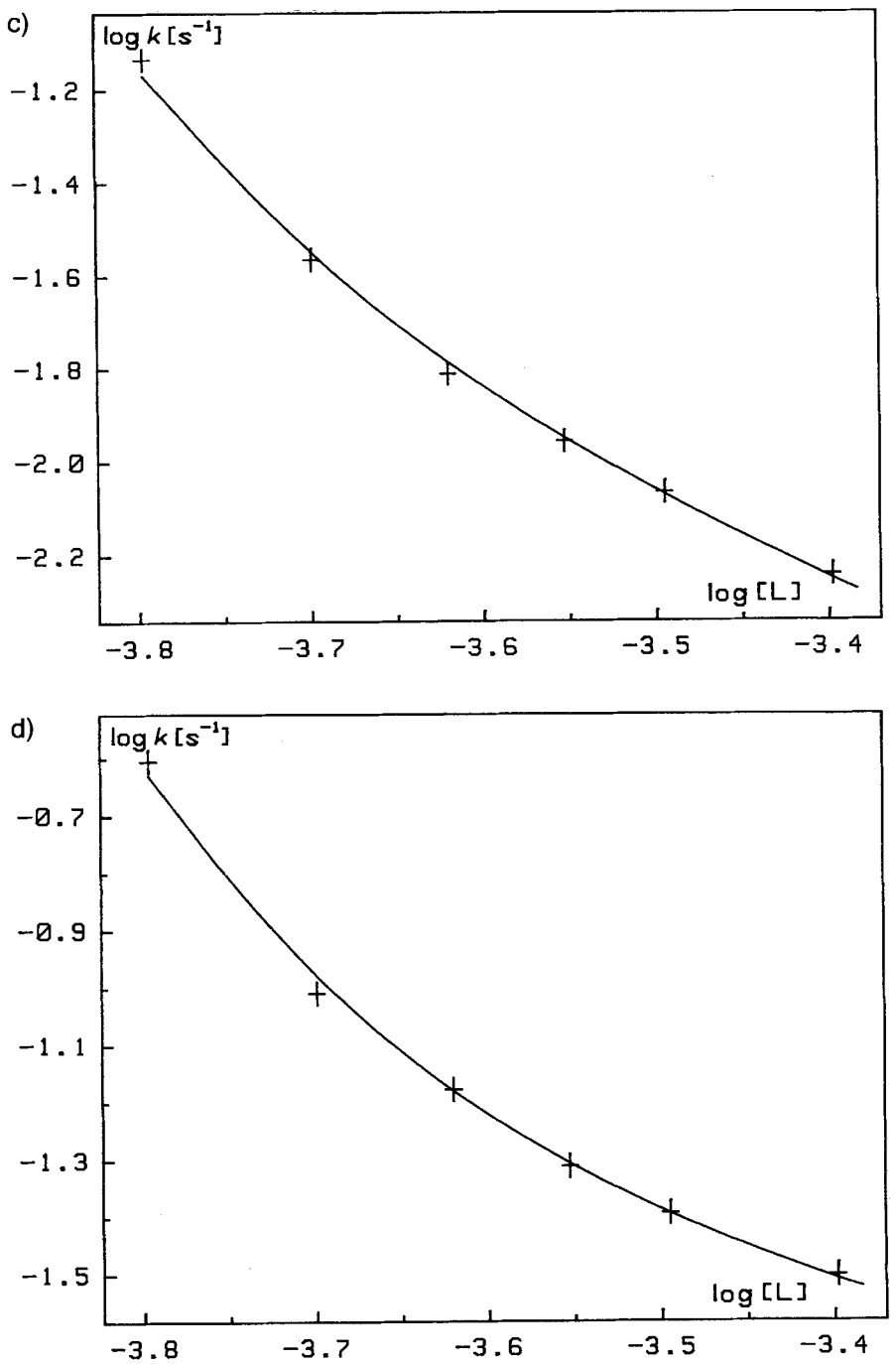

c)
log k [s⁻¹]
-1.2
-1.4
-1.6
-1.8
-2.0
-2.2
log [L]
-3.8
-3.7
-3.6
-3.5
-3.4
d)
log k [s⁻¹]
-0.7
-0.9
-1.1
-1.3
-1.5
log [L]
-3.8
-3.7
-3.6
-3.5
-3.4

Fig. 5 (cont.)

Scheme 3

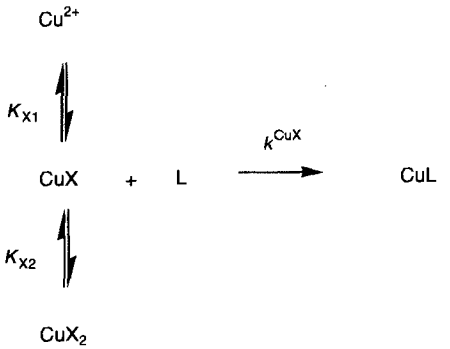


Table 4. *Conditional Rate Constants* (pH 6.5) *for the* Cu^{2+} *Incorporation into* **1**–**4** *in the Presence of X* (nitroso-R salt) *at 25° and* I = *0.1*M

	1	**2**	**3**	**4**
k^{CuX} [$M^{-1}s^{-1}$]	$4.6(1) \cdot 10^3$	$10.6(6) \cdot 10^3$	$4.0(2) \cdot 10^3$	$13.1(5) \cdot 10^3$

rnase, **3** and **4**, are not very different, which is interesting in regard to the 'post-labeling' technique. Worth pointing out is, however, the difference of a factor 3 between **3** and **4** which might be due to the different overall charge of the protein: **3** and **4** have isoelectric points of 4.71 and 9.45 [16], respectively, **3** is, therefore, negatively charged at pH 6.5, whereas **4** carries a positive charge at the same pH.

In conclusion, we can say that the reactivity of the macrocycle towards Cu^{2+} does not drastically depend whether it is a free ligand or conjugated to a protein, and that under typical conditions of the 'post-labeling' technique the incorporation can be done in a relatively short time. The use of an additional ligand to keep the metal ion in solution at pH 6.5 is also helping to increase the rate of incorporation.

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