Solution- and Crystal-Phase Covalent Modification of Lysozyme by a Purpose-Designed Organoruthenium Complex. A MALDI-TOF MS Study of its Metal Binding Sites

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Study of the reaction between the transition organometallic complex 4-ruthenocenyl 2,6-dimethylpyrylium tetrafluoroborate and the enzyme hen egg white lysozyme (HEWL) in solution and by diffusion in crystals was performed by use of a combination of spectroscopic and chromatographic methods. Conjugation involving the lysine residues of lysozyme appeared to occur readily, yielding very stable ruthenocenyl pyridinium adducts with average degrees of incorporation ranging from 0.2 to 1.8 metal complexes per protein molecule, depending on reaction conditions. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) revealed that the protein conjugates were in fact mixtures of unmodified, mono-, di- and sometimes tripyridinium adducts. In combination with reversed-phased HPLC, we were able to show that six different monoruthenocenyl pyridinium adducts were formed in solution. This result was confirmed by trypsin digestion of a ruthenocenyl pyridinium conjugate and MALDI-TOF MS analysis of the peptide mixture, which showed that lysines 1, 13, 33, 96, 97 and 116 were involved in the reaction with the pyrylium complex, lysines 13, 33 and 116 being the major binding sites. In the tetragonal crystal state, no binding of the ruthenium complex was shown to occur at lysine 116, owing to steric hindrance at this particular position.

Introduction

One of the main hurdles to overcome in the course of X-ray structural analysis of proteins is the so-called "phase problem": the phasing of X-ray diffraction data. Several well known approaches for addressing this issue are currently available,[1, 2] in particular molecular replacement and multiple isomorphous replacement (MIR) methods. The latter method remains predominant for the resolution of de novo structures.[3] The MIR approach requires the preparation of at least two heavy atom derivatives: that is, protein crystals in which heavy elements are covalently or non-covalently bound at well defined positions within the crystal lattice, and collection of their X-ray diffraction data together with the native crystals.[4] To be useful for structural determination, these heavy atom derivatives should firstly be isomorphous with the native crystals: that is, no alteration of the packing and no change of the unit cell dimensions should occur after binding. Secondly, the number of heavy atom binding sites should be low (ideally close to one) to facilitate the determination of their position on difference Patterson maps. Finally, the fractional increase in intensity of the diffracted beams caused by the binding of the heavy atom(s) should be much higher than the error margin of the measured intensities. Crick and Magdoff have shown that the fractional change in intensity ΔI can be expressed as:

$$\Delta I = \sqrt{2} \times \sqrt{\frac{N_E}{N_P}} \times \frac{Z_E}{Z_P} \quad (1)$$

with N_E the number of atoms with atomic number Z_E in a unit cell containing N_P protein atoms with a mean atomic number Z_P.[5] The ideal situation occurs when one heavy atom is bound to every protein at the same site in the crystal. If this is not the case (occupancy <1), Z_E has to be reduced in proportion to the number of protein molecules having a heavy atom associated with them. For all these reasons, while a wide range of reagents is available to the crystallographer,[6] finding the most useful one is generally tedious, mostly because their reactivity is not predictable.[7] This is why improvements in heavy element

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phasing are actively sought after, with particular interest in the development of new heavy atom derivatives.[8–16]

In the last few years, we have been undertaking the design of organo-complexes of heavy transition metals targeted towards protein amino groups, to produce covalently bound heavy atom derivatives.[17–21] Most importantly, transition organometallic complexes of the pyrylium ion series appeared particularly well suited to the introduction of a transition metal into protein crystals in a side chain selective manner, owing to their solubility in water, wide pH range of reactivity, slow hydrolysis rate and ease of spectroscopic detection of the conjugation.[22, 23] For example, two organometallic pyrylium ions bearing an arene chromium tricarbonyl moiety were recently used to introduce chromium atoms into crystals of the enzyme hen egg white lysozyme (HEWL) without loss of crystal isomorphism.[11] This protein is well suited to test new heavy atom reagents as it is commercially available at 95% purity, possesses a relatively small number of potential reaction sites and is readily crystallized under a large variety of conditions and crystal lattices.[24]

However, chromium is not heavy enough to be of any real utility for protein X-ray structural analysis. For example, when the equation of Crick and Magdoff is applied to the relatively small protein HEWL hypothetically derivatized with one chromium atom per protein molecule with an occupancy of one, the calculated increase in intensity would only be 15%. Moreover, the previously synthesized 4-([ηç^5-cyclopentadienyl)tricarbonylrhenium]) 2,6-diphenylpyrylium salt is insoluble in water. We have therefore designed a new, water-soluble pyrylium salt bearing two methyl substituents and the transition metal ruthenium in the form of a ruthenocenyl moiety. Here we report studies of the reaction between this complex and HEWL, carried out both in solution and by diffusion in crystals. We show that MALDI-TOF MS coupled with RP-HPLC showed unambiguously that chemical modification of the enzyme occurred under both sets of conditions and that ruthenocenyl pyridinium conjugates were formed. The degree of incorporation of ruthenocenyl groups into HEWL was also evaluated and, in combination with trypsin digestion, the preferential binding sites were determined.

Results

Preparation of the lysozyme conjugates and spectroscopic characterization

Known amounts of HEWL and ruthenocenyl pyrylium salt **1** were combined in a buffer solution at controlled pH (around neutrality) and compounds were allowed to react at room temperature for between 17 hours and 9 days. Reaction conditions were as close as possible to those applied to protein crystals. The unbound organometallic complex was eliminated by gel filtration chromatography. The resulting deep yellow protein sample was then analysed by UV/Vis spectrometry. A typical spectrum of the protein conjugate is shown in Figure 1, together with the spectra of lysozyme and of 4-ruthenocenyl *N*-butyl-2,6-dimethylpyridinium tetrafluoroborate (**2**) prepared by treatment of **1** with *n*-butylamine. The lysozyme displayed an absorption maximum at 280 nm, typical of its aromatic amino acids, whereas the protein conjugate displayed one extra maximum at 399 ± 2 nm and a shoulder at 312 ± 2 nm. These two features were also present in the absorption spectrum of **2**, indicating that a pyridinium conjugate was most probably formed by reaction of lysozyme with **1**. These two relatively low-energy bands are characteristic of conjugated ferrocenyl and ruthenocenyl complexes with potent (hetero)cyclic acceptors such as tropylium,[25] benzopyrylium and pyridinium[26] cations. These organometallic chromophores have received a great deal of attention thanks to their NLO properties. From literature precedent,[24] the higher-energy band for **2** was assigned to the $\pi - \pi^*$ (metal to ligand) transition, and the lower one to the MLCT transition. From this set of spectra, the average number of ruthenocenyl pyridinium groups per protein molecule, also called the coupling ratio (CR), was determined. Results for four different samples are reported in Table 1.

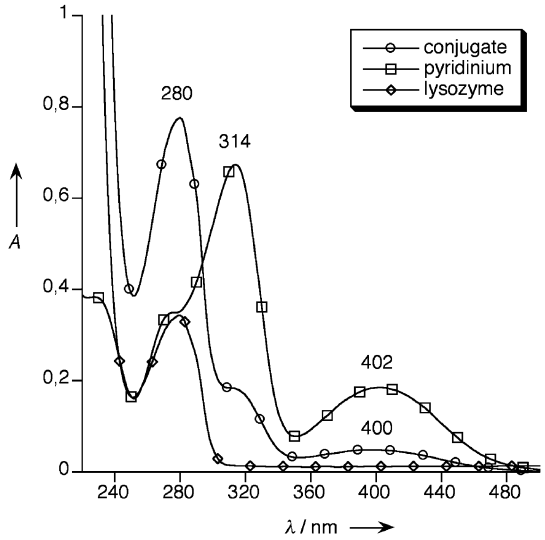

Figure 1. *Superimposition of the UV/Vis spectra of lysozyme, compound **2** and a conjugate resulting from the reaction of **1** with HEWL.*

These samples were subjected to MALDI-TOF MS analysis, together with the unmodified protein. The corresponding mass spectra, restricted to the region of singly charged species, are shown in Figure 2. HEWL displayed one major peak at *m/z* 14314 ± 5, in agreement with the mass calculated from the published primary sequence of hen egg white lysozyme.[27] In addition, minor peaks were observed at *m/z* 14479 and 14532; these may be assigned to lysozyme adducts with impurities and/or matrix-related species. Other peaks in the *m/z* 7000 spectral

Table 1. *Characterization of HEWL-ruthenocene conjugates. Comparison of UV/Vis, HPLC and MALDI-TOF MS data.*[a]

Sample	Conditions of reaction	Average CR[b]	Relative amount of unmodified protein[c] [%]	Relative abundance of the peak at $m/z = 14314$ [%]
1	[HEWL] = [**1**] = 500 μM, KH_2PO_4 25 mM, NaCl 0.8 M pH 6.8, 17 h, RT	0.2	83	77 ± 3
2	[HEWL] = [**1**] = 500 μM, HEPES 0.1 M, pH 7.5, 9 d, RT	0.7	44	56 ± 3
3	[HEWL] = [**1**] = 500 μM, KH_2PO_4 50 mM pH 6.8, 7 d, RT	0.9	33	50 ± 3
4	[HEWL] = 500 μM, [**1**] = 1000 μM, KH_2PO_4 50 mM pH 6.8, 7 d, RT	1.8	15	40 ± 2

[a] See corresponding mass spectra in Figure 2. [b] Coupling ratio, $CR = (38000 \times A_{400})/(3950 \times A_{280} - 7800 \times A_{400})$. [c] Calculated from RP-HPLC data.

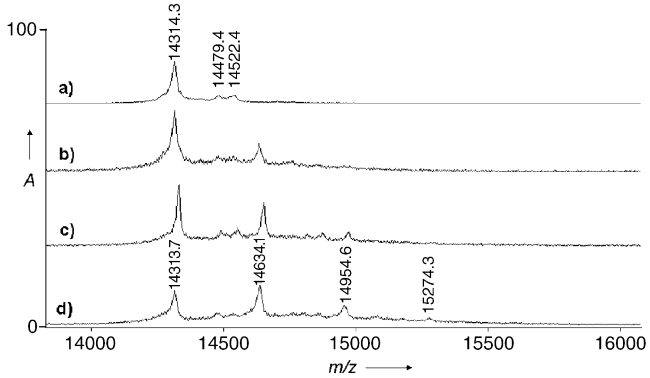

Figure 2. *MALDI-TOF mass spectrum of a) HEWL, b) sample #1, c) sample #2, d) sample #4. Matrix: 3,5-dimethoxy-4-hydroxycinnamic acid (SA).*

region were also present in the mass spectrum, corresponding to doubly charged species (results not shown). The mass spectrum of all the lysozyme conjugates (i.e., resulting from the gel filtration of the reaction mixtures) displayed a series of well defined peaks, one at m/z 14314 ± 5 together with one, two or three extra peaks separated from each other by 320 ± 2 u. This incremental mass increase corresponded to the successive conversion of one, two or three primary amines of HEWL into one, two or three 4-ruthenocenyl 2,6-dimethylpyridinium groups. The resulting increase in charge was compensated for by removal of protons from other parts of the molecule, so that the singly charged species were observed each time. Such processes involving proton loss are often observed during MALDI-TOF mass analysis of species cationized by doubly (or triply) charged cations.[28, 29] No other peaks in this m/z range were observed (except for the minor adducts described above), indicating that reaction between lysozyme and **1** solely provided pyridinium conjugates. The relative intensity of the monoconjugate, diconjugate and triconjugate singly charged mass peaks appeared to increase with time, whereas the relative intensity of the unmodified protein peak seemed to decrease (Table 1). However, the MS results do not allow evaluation of the coupling ratio since the ion yields of the different species can differ.

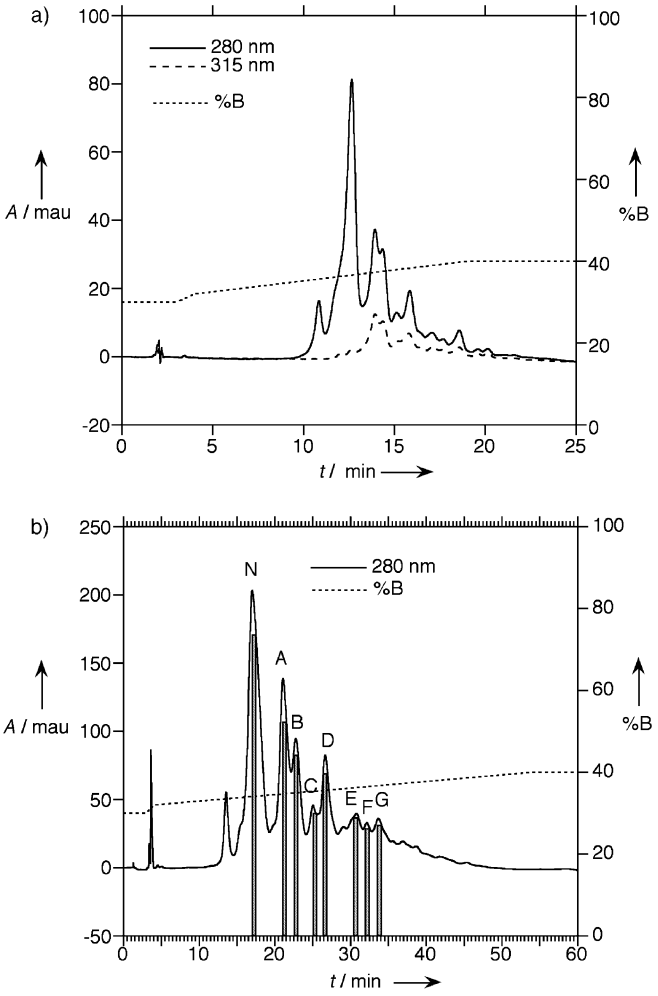

Figure 3. *RP-HPLC of a) sample #2; b) sample #3. The column was Vydac 214 TP (5 μm, 4.6 × 150 mm). Linear gradient of CH_3CN/0.1% TFA in water/0.1% TFA as indicated on the graph. Flow rate 1 mL min^{-1}. Peak detection at 280 and 315 nm.*

Reversed-phase HPLC of lysozyme conjugates

The HEWL conjugates were further analysed by RP-HPLC. A typical chromatogram (sample #2) is shown in Figure 3a. On the analytical scale, each of the chromatograms displayed one major peak at ca. 12 min, readily identified as corresponding to the unmodified protein. In addition, several other poorly resolved peaks appeared after 13 min, corresponding to more hydrophobic proteic species. As these species also absorbed at 315 and 400 nm (unlike the native protein, which only absorbs up to 300 nm; see spectrum in Figure 1), they were readily identified as HEWL-ruthenocenyl pyridinium adducts. The number and relative amount of these adducts appeared to depend on the reaction conditions. For example, the relative amount of

unmodified lysozyme in the mixtures was roughly evaluated from the relative 12 min retention time peak area, and data are reported in Table 1. On the whole, these mixtures of adducts seemed to be more heterogeneous than those resulting from the reaction of (4-benzene chromium tricarbonyl) pyrylium ions[11] or metallo-carbene complexes.[19] This might indicate that a greater number of protein sites were involved in the reaction with **1**. Moreover, under the same elution conditions, the ruthenocenyl pyridinium adduct species were less well separated than the metallo-aminocarbene conjugate species, primarily because the positive charge of the amine-bearing residue after reaction is conserved in the first case whereas the aminocarbene residue is neutral, and therefore more hydrophobic.

Reversed-phase HPLC was further performed on a semi-preparative scale on sample #3 (Figure 3b) with use of a shallower gradient slope. Fractions N and A–G were collected separately and analysed by MALDI-TOF MS (Table 2, Figure 4). As

Table 2. *Characterization of HEWL-ruthenocene adducts fractioned by reversed-phase HPLC (sample #3). Calculation of the number of ruthenocenyl groups EC bound per protein from HPLC peak areas and analysis of the fractions by MALDI-TOF MS.*[a]

Chromatographic fraction (retention time)	A_{280}/A_{315}	EC	Singly charged mass peaks observed at *m/z*
N (17 min)	–	0	14314
A (21.1 min)	2.85	1.1	14634
B (22.8 min)	2.99	1.1	14634
C (25 min)	2.95	1.1	14634, 14954
D (26.6 min)	1.96	1.8	14634, 14954
E (30.7 min)	2.36	1.4	14634, 14954
F (32.1 min)	1.97	1.8	14954
G (33.7 min)	2.27	1.5	14954

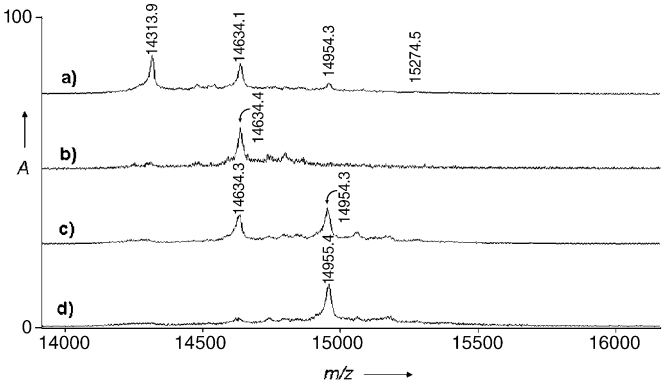

Figure 4. *MALDI-TOF mass spectrum of a) sample #3, b) fraction A, c) fraction D, d) fraction F. Matrix: SA.*

expected, fraction N gave a molecular ion at $m/z = 14314$, corresponding to the molecular mass of lysozyme. Fractions A, B, C and G each gave a molecular ion at $m/z = 14634$ ($M+320$ u), corresponding to a singly charged monoruthenocenyl pyridinium adduct; fractions D and E each gave two molecular ions at $m/z = 14634$ and 14954 ($M+640$ u), corresponding to a mixture of singly charged mono- and diruthenocenyl pyridinium adducts, and finally species F gave a single peak at $m/z = 14954$, corresponding to a diruthenocenyl pyridinium adduct. Incidentally, the presence of these single peaks indicates that the ruthenocenyl pyridinium group bound to lysozyme was not labile under the conditions of MALDI-TOF MS analysis. Thus at least six different monoconjugated proteins (two major and four minor adducts) and four different diconjugated species were identified in sample #3 fractionated by RP-HPLC. However, the elution conditions did not allow a complete separation of all the species formed by the reaction of **1** with HEWL. A much shallower gradient slope permitted full resolution of all the species (result not shown).

On the other hand, the extent of conjugation (EC) of each of the reversed-phase HPLC fractions was evaluated from the ratio of the peak areas at 280 and 315 nm by application of the following equation:

$$EC = \left(\frac{\varepsilon_{280}^{HEWL} + \varepsilon_{280}^{2}}{\varepsilon_{315}^{2}}\right) \times \left(\frac{A_{315}}{A_{280}}\right) \quad (2)$$

From the calculations (Table 2) it appeared that the EC of fractions A, B, C, E and F was in agreement with the mass spectrometric determinations, while results differed for fractions D (diruthenocenyl adduct for the EC calculation) and G (mixture of mono- and diruthenocenyl adducts for the EC calculation). We note, however, that the EC calculation was based on complete peaks, while only the part of the HPLC peaks depicted by the shaded boxes in Figure 3B was actually collected.

On the whole, it appears that the fractions separated on the reversed-phase column contained either the unmodified protein or some ruthenocenyl pyridinium adducts. With use of a relatively shallow gradient slope, at least four of these fractions appeared to contain pure species while the others evidently contained mixtures of protein adducts.

Digestion of lysozyme conjugates by trypsin

The enzyme hen egg white lysozyme is a single peptide chain of 129 amino acids including seven primary amines, six of these being the ε-amino groups of the lysine residues (Lys1, Lys13, Lys33, Lys96, Lys97 and Lys116) and one being the α-amino group of the N-terminal residue. To determine which of these functional groups were involved in the reaction of lysozyme with **1**, the protein conjugate sample #2 was subjected to proteolysis by trypsin after reduction of the disulfide bridges and carboxymethylation of the resulting cysteines with iodoacetic acid.

The mixture of peptides was analysed by MALDI-TOF MS (Figure 5, Table 3). The peptide mass map resulting from the tryptic proteolysis of sample #2 contained a total of 24 peaks, corresponding to singly charged peptide species as reflected by the isotopic cluster. Eight of them were assigned to the expected tryptic peptides generated by cleavage of the peptide bond at the C-terminal side of the unmodified basic residues and gave a sequence coverage of 77%. Measured masses fitted well with the masses calculated from the peptide sequence. The detection of peptide (115–125) indicated that the bond between Lys116

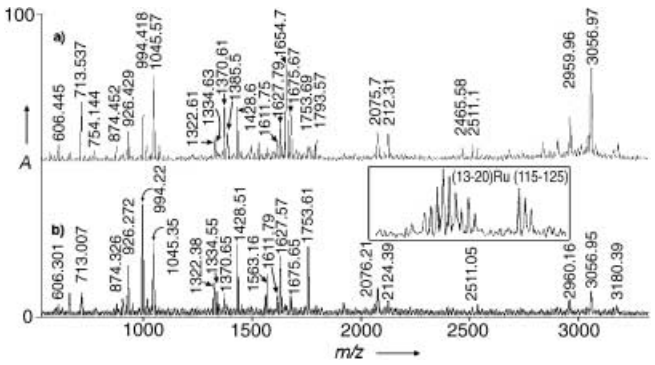

Figure 5. *MALDI-TOF mass spectrum of the mixture of peptides resulting from the tryptic proteolysis of a) sample #2, b) HEWL crystal soaked in* ***1*** *(0.75 mM) in HEPES buffer (0.1 M pH 7.5 containing 0.8 M NaCl) for 3 months. Matrix: α-cyano-4-hydroxycinnamic acid (HCCA).*

Table 3. *Tryptic digestion of lysozyme (crystal) and lysozyme ruthenocenyl pyridinium conjugates (sample #2 and crystal soaked for 3 months). MALDI-TOF MS analysis of the peptide mixtures.* m/z *correspond to mono-isotopic values for unmodified peptides and average values for modified peptides.[e]*

In solution	In the crystal state		Calcd	Peptide	Labelling site
	untreated	soaked			
$m/z \pm 0.2$	$m/z \pm 0.2$	$m/z \pm 0.2$	$m/z \pm 0.2$		
606.4	606.3	606.3	606.36	(1 – 5)	
874.4		874.3	874.41	(15 – 21)	
–[d]	1326.6	–[d]	1326.6	(22 – 33)[b]	
1428.6	1428.5	1428.5	1428.64	(34 – 45)	
1627.8	1627.6	1627.6	1627.78	(32 – 45)[a]	
1753.7	1753.6	1753.6	1753.83	(46 – 61)	
994.4	994.3	994.2	994.38	(62 – 68)[b]	
2511.1		2511	2511.13	(74 – 96)[b]	
1675.7	1675.6	1675.6	1675.80	(98 – 112)	
1334.6	1334.5	1334.5	1334.65	(115 – 125)[b,c]	
1045.6	1045.4	1045.3	1045.54	(117 – 125)	
926.4		926.3	926.11	(1 – 5)Ru	Lys1
713.5		713	712.9	(1 – 3)Ru[a]	Lys1
1370.6		1370.6	1370.63	(6 – 14)Ru[b]	Lys13
754.1			753.94	(12 – 14)Ru[a]	Lys13
1322.6		1322.4	1322.47	(13 – 20)Ru[a]	Lys13
1793.6			1794	(22 – 34)Ru[a,b]	Lys33
3057		3057	3057.32	(22 – 45)Ru[b]	Lys33
1611.7		1611.8	1611.82	(97 – 108)Ru[a]	Lys97
2960		2960.2	2960.37	(74 – 97)Ru[b]	Lys96
2124.3		2124.4	2124.43	(97 – 112)Ru	Lys97
1654.7			1654.9	(115 – 125)Ru[b]	Lys116

[a] Chymotryptic cleavage. [b] Carboxymethylated. [c] Partial tryptic cleavage. [d] Masked by the (13 – 20)Ru peak. [e] See corresponding mass spectra in Figure 5.

also observed, resulting in the formation of peptides (1 – 3)Ru, (13 – 20)Ru, (22 – 34)Ru and (7 – 108)Ru, which were clearly identified on the tryptic map. The three other ruthenium-containing peptides could not be identified from their measured molecular masses.

As peptides (6 – 14)Ru, (22 – 45)Ru, (97 – 112)Ru and (115 – 125)Ru each included a single lysine residue, they were unequivocally assigned to labelling at lysines 13, 33, 97 and 116, respectively. No tryptic cleavage of the peptide bond after these lysines occurred, as generally observed when the ε-amino group of lysine residues is chemically modified.[30] Peptide (74 – 97)Ru contained Lys96 and Lys97, but as we have just seen that the presence of a ruthenocenyl pyridinium entity on the lysine side chain inhibited the hydrolysis of the adjacent peptide bond, it was unambiguously assigned to labelling at Lys96. Finally, peptide (1 – 5)Ru corresponded to labelling at Lys1, but MALDI-TOF MS alone did not allow us to distinguish between labelling at the α-amino group or at the ε-amino group of this residue. An Edman sequencing experiment performed on this purified peptide should in principle resolve this uncertainty. Interestingly, while neither peptide (6 – 13) nor peptide (6 – 14) were observed on the peptide mass map of lysozyme, the corresponding labelled peptide (6 – 14)Ru was clearly observed and was in fact one of the most intense peaks of the spectrum.

From these data we can first conclude that all the possible binding sites of lysozyme (i.e., the six lysine residues) were involved in the reaction with complex **1** in solution. In other words, no reaction selectivity of **1** towards one particular lysine residue of HEWL was observed. Secondly, no binding site was fully modified, in agreement with the presence of some unmodified protein in the conjugate sample, as shown both by MALDI-TOF MS (Figure 2) and by RP-HPLC (Table 1). Finally, it was not possible to determine the preferential sites of reaction from the absolute intensities of the mass peaks of the ruthenocenyl peptides, as there is no direct relationship between peak intensity and proportion of the related peptide in the mixture, possibly due to different ion yields.

and Gly117 was only partially hydrolysed. The other 13 peaks corresponded to peptides carrying one ruthenium atom as seen by their characteristic isotopic cluster (see inset in Figure 5). Most of the peaks were readily assigned to ruthenocenyl pyridinium containing peptidic fragments by first subtracting 320 (that is, the molecular mass of the ruthenocenyl dimethylpyridinium group) from the measured value. Peptides (1 – 5)Ru, (6 – 14)Ru, (22 – 45)Ru, (74 – 97)Ru, (97 – 112)Ru and (115 – 125)Ru bearing one ruthenocenyl pyridinium group were identified on the tryptic mass map and corresponded to tryptic cleavages. Chymotryptic cleavages after several aromatic residues were

The mixture of tryptic peptides resulting from the proteolysis of sample #3 (which was very close in composition to sample #2) was analysed by RP-HPLC (detection set to 225 and 400 nm, Figure 6). The reversed-phase chromatogram of the tryptic peptide mixture recorded at 225 nm displayed several peaks

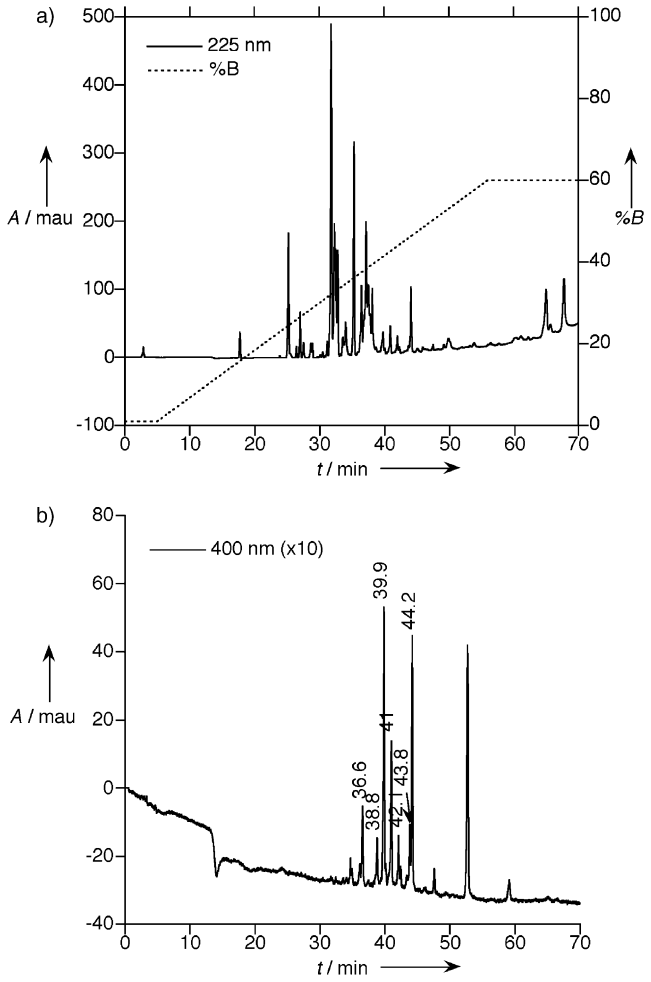

Figure 6. RP-HPLC of the mixture of peptides resulting from the tryptic proteolysis of sample #3. The column was Macherey Nagel C18 HD (5 μm, 3 × 250 mm). Linear gradient of CH_3CN/0.1% TFA in water/0.1% TFA as indicated on the graph. Flow rate 0.5 mL min^{-1}. Peak detection at 225 (a) and 400 nm (b).

between 18 and 45 min retention time, corresponding to the set of peptides resulting from proteolysis. When the HPLC effluents were monitored at 400 nm (where only the ruthenocenyl pyridinium chromophore absorbs), the chromatogram displayed a total of seven major peaks between 36 and 45 min retention time that either coeluted with the most hydrophobic peptides or later. Three of them were intense (at 39.9, 41.0 and 44.2 min) and the other four (36.6, 38.8, 42.1 and 43.8 min) were weaker. If one chromatographic peak corresponds to a single peptide, this might indicate that there are three major and four minor ruthenocenyl-labelled products, which would correspond to three major sites of reaction and four minor sites.

To examine this hypothesis, the peaks absorbing at 400 nm were collected separately and six of them were analysed by MALDI-TOF MS (Table 4, Figure 7). The 39.9, 41.0 and 44.2 min retention-time peaks were unequivocally assigned to the ruthenocenyl pyridinium peptides (6 – 14)Ru + (97 – 112)Ru, (115 – 125)Ru and (22 – 45)Ru, respectively, corresponding to labelling at Lys13, Lys97, Lys116 and Lys33. The 36.6 and 43.8 min retention time peaks were seen to correspond to mixtures of two

Table 4. *MALDI-TOF MS analysis of tryptic peptides fractionated by RP-HPLC.*[a]

Retention time [min]	$m/z \pm 0.2$	Peptide	Labelling site
36.6	1322.3	(13 – 20)Ru[b]	Lys13
	1370.6	(6 – 14)Ru	Lys13
38.8	926.0	(1 – 5)Ru	Lys1
39.9	1370.6	(6 – 14)Ru	Lys13
	2124.2 (weak)	(97 – 112)Ru	Lys97
41.0	1654.8	(115 – 125)Ru	Lys116
43.8	712.8	(1 – 3)Ru[b]	Lys1
	2960	(74 – 97)Ru	Lys96
44.2	3056.9	(22 – 45)Ru	Lys33

[a] *m/z* correspond to average values. See corresponding mass spectra in Figure 7. [b] Chymotryptic cleavage

ruthenocenyl peptides corresponding to labelling at Lys13, Lys1 and Lys96. Curiously, ruthenocenyl pyridinium peptide (6 – 14)Ru was detected in two different and well separated fractions. In the course of our previous work on tungsten pentacarbonyl amino-carbene conjugates of lysozyme, similar behaviour was observed for peptide (6 – 14)W.[19] No satisfactory explanation was found to explain this unusual behaviour. Finally, the 38.8 min retention time peak was assigned to peptide (1 – 5)Ru; that is, labelling at Lys1.

Overall, eight of the thirteen labelled peptides initially detected in the mixture were identified in the RP-HPLC fractions. The major chromatographic peak at 400 nm (retention time: 39.9 min) contained the two ruthenocenyl pyridinium peptides (6 – 14)Ru and (97 – 112)Ru. The intensity of the mass peak of the latter being much weaker than that of the former both for the mixture of tryptic peptides and for the RP-HPLC fraction, we expect that the major species eluting at 39.9 min is peptide (6 – 14)Ru containing the ruthenocenyl pyridinium group at Lys13. Eventually, lysines 13, 33 and 116 appeared as major sites of reaction whereas lysines 1, 96 and 97 were minor sites of reaction in solution.

Crystal soaking experiments

Lysozyme crystals were grown by the classical hanging drop method, by use of 0.8 M NaCl as precipitant in acetate buffer (pH 4.5). Under these conditions, large, tetragonal crystals grew within one week. Some of these crystals were transferred into a drop of potassium phosphate (pH 6.8) or HEPES (pH 7.5) containing 0.8 M NaCl. After equilibration, a drop of a solution of **1** in the same buffer was added to the crystals, which were left to soak for between 1 day and 3 months over the same reservoir solution. Crystals were removed from the drop and some were rinsed by transferring them several times into a fresh drop of reservoir solution. All the crystals (rinsed and not rinsed) were pale to deep yellow after this step. Once dissolved, the crystals were analysed by MALDI-TOF MS. The resulting mass spectra (not shown) had the same quality as those obtained from the protein labelled in solution shown in Figure 2. The spectrum of the slightly yellow HEWL crystal soaked for one day was identical to those of the untreated crystal and the protein in solution

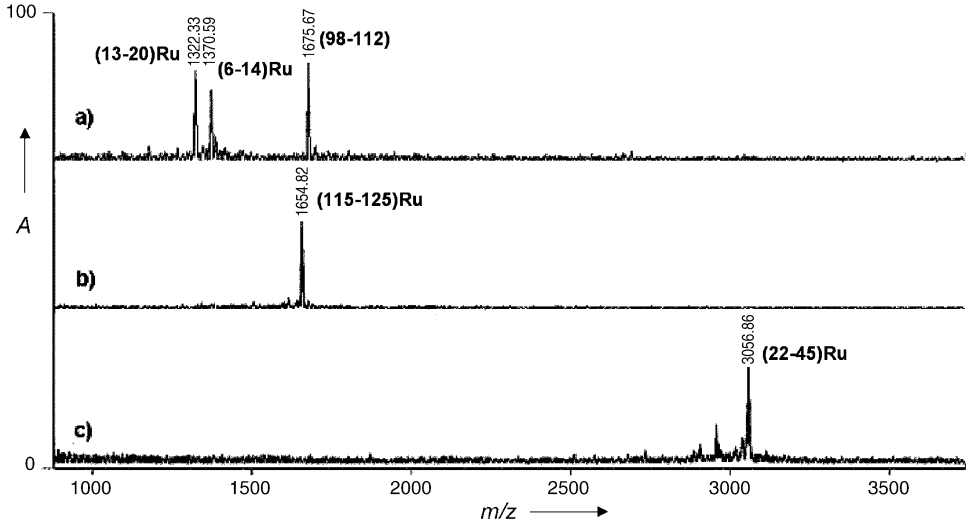

Figure 7. MALDI-TOF mass spectra of RP-HPLC fractions resulting from the tryptic proteolysis of sample #3. a) $t_R =$ *36.6 min, b)* $t_R = 41$ *min, c)* $t_R = 44.2$ *min. Matrix: HCCA.*

(peak at $m/z = 14314$). After seven days, a peak corresponding to the monoruthenocenyl pyridinium conjugate appeared (accounting for 26% of the mixture). After 30 days, the intensity of this peak increased to 37% of the mixture and a second peak corresponding to the diruthenocenyl pyridinium conjugate appeared (11%). Finally, the crystal soaked for three months was not fully soluble in water and the soluble part was shown to contain some monoruthenocenyl pyridinium adducts (42%) together with diruthenocenyl pyridinium adducts (25%) and triruthenocenyl pyridinium adducts (7%) within the experimental error of measurement. These values are only indicative and cannot be related to the relative amount of each species in the conjugates, as previously mentioned.

Thus treatment of lysozyme with **1** proceeded in the crystal state as in solution, leading solely to ruthenocenyl pyridinium species. The reaction appeared to proceed more slowly, probably because the reagent needs to diffuse in the water channels in order to reach the reactive amino groups of the protein. Back-diffusion of the excess reagent by soaking crystals in the reservoir solution caused no change whatsoever in the spectrum of the crystal. This means that MALDI-TOF MS is a very powerful method to monitor the chemical modification of the protein crystal even in the presence of a high concentration of precipitant and heavy metal reagent, as previously observed.[31] It is also a very sensitive method (amount of compound measured approximately 0.7 nmol).

The last step of this study was to determine which were the protein sites that carried the ruthenocenyl pyridinium group in one of the treated crystals. After partial dissolution in water, the lysozyme crystal soaked for three months was subjected to tryptic proteolysis (after reduction of the disulfide bridges and carboxymethylation of the resulting cysteines by a simplified procedure) and the mixture of peptides was directly analysed by MALDI-TOF MS (Figure 5, Table 3). For comparison, the same procedure was applied to an untreated crystal. The peptide mass map of an untreated lysozyme crystal displayed a total of eight peaks corresponding to a sequence coverage of 74%. The sequence coverage increased to 90% for the treated crystal and 12 extra peaks corresponding to peptides containing one ruthenium atom were now detected. Ruthenocenyl pyridinium peptides (1–5)Ru, (6–14)Ru, (22–45)Ru, (74–97)Ru and (97–112)Ru were unambiguously identified from the molecular mass measurements as before. Three other peaks were identified with peptides generated by chymotryptic proteolysis, and the three remaining other peaks were unidentified. Most noticeably, peptide (115–125)Ru was missing in the peptide mass map, indicating that no reaction occurred with lysine 116 in the crystal state (reaction did occur in solution; see above). Conversely, Lys1, Lys13, Lys33, Lys97 and Lys96 were involved in the reaction with **1** in the crystal state. Again, the presence of all the unlabelled peptides indicates that none of these positions was fully modified. The order of mass peak intensities for the ruthenium-containing tryptic peptides was as follows: (1–5)Ru > (6–14)Ru = (22–45)Ru > (74–97)Ru > (97–112)Ru. Assuming an identical response (detection efficiency) for the solution and crystal experiments, we may infer that position Lys1 was modified to a greater extent in the crystal phase than in solution. However it should be borne in mind that the ionization yields of all the peptides (unlabelled and labelled) may differ substantially; therefore the intensity of the mass peaks is not related to the relative amount of each peptide in the mixture.

Discussion

Pyrylium ions are heterocyclic aromatic compounds with a marked electrophilic character at the α and γ positions of the ring. The conversion of substituted pyrylium salts into pyridinium salts in the presence of primary amines is a well known reaction and its mechanism long established.[32] Furthermore, the extension of this reaction to proteins is well documented in the literature[33–35] and was shown to proceed similarly.[36] We have previously shown by UV/Vis spectroscopy that pyrylium ions carrying a cyclopentadienyl manganese (rhenium) tricarbonyl group at the *para* position were able to react with the protein bovine serum albumin (BSA) to form protein pyridinium conjugates.[22] More recently, two (4-benzenechromium tricarbonyl) 2,6-dimethylpyrylium derivatives were also shown to yield pyridinium adducts of BSA in solution[23] and of HEWL in solution and by diffusion in crystals[11] as evidenced by IR spectroscopy.

IR spectroscopy not being available in this case because of the absence of a metal carbonyl probe,[37] MALDI-TOF mass spectrometry proved to be the best method to show the formation of ruthenocenyl pyridinium adducts of HEWL both in solution and by diffusion of **1** in single crystals. Indeed, this method has been successfully applied to measure the degree of derivatization of hapten-carrier protein conjugates[38–40] and fluorescein-labelled proteins[41, 42] and to determine the preferential binding sites. Mass spectrometry either in the ESI or the MALDI-TOF mode can considerably improve the screening process for identification of the suitable heavy atom reagents[31] and conditions for their introduction into protein crystals.[43, 44] MALDI-TOF MS nonetheless appears better suited to the analysis of protein crystals, because this method is highly tolerant of additives used in protein crystallization in comparison with ESI-MS.[45]

The formation of mono-, di- and even trisubstituted adducts in solution and in the crystal phase, together with the presence of some unmodified protein, was readily evidenced by MALDI-TOF MS. However, the relative amount of unmodified protein in the protein conjugates cannot be calculated from the relative intensity of the corresponding mass peak, due to possible different ionization yields of the various species.

Six different monosubstituted adducts, together with four different disubstituted adducts, were identified by mass spectroscopic analysis and differential UV/Vis spectroscopy, after RP-HPLC fractionating of a lysozyme conjugate prepared in solution. This finding may be explained in terms of the reaction of six amino groups of HEWL and fits well with the seven potential binding sites (six lysines and the α-amino group of the N-terminal residue).

The large heterogeneity of the protein conjugates was confirmed by tryptic digestion coupled with mass spectrometric analysis. This experiment enabled us to show that all the lysine residues of HEWL were indeed involved in the reaction with **1** in solution. Separation of the labelled tryptic peptides by HPLC was necessary in order to identify that the preferential binding sites for **1** in solution were lysines 13, 33 and 116. Examination of the X-ray tetragonal crystal structure of lysozyme suggested that the areas of the seven amino groups of lysozyme exposed to solvent ranged from 5 Å^2 (Lys1, Nα) to 40 Å^2 (Lys33; Table 5).[46] Calculation of the surface accessibility of the lysozyme amino groups from high-resolution X-ray data (PDB file 193L) by use of the CSU software[47] confirmed that, except for the N-terminal amine, they were all almost identically accessible to solvent and reagents. The ε-amino groups of lysines 13, 33 and 116 appeared to be involved in hydrogen bonds with the carbonyl of Leu129, the δ-carbonyl of Asn37 and the δ-carbonyl of Asn106, respectively. In any case, the residues of HEWL exposed to solvent are known to be highly mobile in solution.[48] Comparison with literature reports indicated that acetylation of HEWL with acetic anhydride occurred preferentially at Lys97 and Lys33,[49, 50] which also display the highest area exposed to solvent (see Table 5). Acylation of HEWL with activated ester derivatives of estrone glucoronide[51] and pregnanediol glucoronide[52] also occurred at Lys33, Lys97, together with Lys116. Interestingly, the hydrophobic amine-targeted reagents $(CO)_5W{=}(OMe)Me$[19] and dinitrofluorobenzene[53] were also shown to react preferentially with Lys13, Lys33 and Lys116. This indicates that the accessibility of the individual amine functions is not the sole parameter that governs their reactivity. Indeed, the accessibility values have been shown to depend on the crystal form taken to calculate them.[53] The pK_a value of the amino groups of lysozyme may play a role, but this hypothesis is difficult to confirm in the absence of comparative experiments run at different pH values. The pyrylium-pyridinium conversion operates by a multi-step mechanism, involving successive base- and acid-catalysed reactions, thus complicating the study of pH effect on the reaction.[32] From the literature results reported above, it appears that the preferential binding sites depend on the reagent. Thus, preferential reaction with lysines 13 and 33 might be explained by a favourable micro-environment around these two residues. On looking carefully at the X-ray structure of the protein, it appears that these two lysines (together with Lys116) are surrounded by hydrophobic amino acids: that is, Leu129, Ala10 and Ile124 for Lys13 and Phe34, Phe38 and Trp123 for Lys33. This might explain why the relatively hydrophobic compound **1** and also the metallo-carbene $(CO)_5W{=}(OMe)Me$ and dinitrofluorobenzene showed a preferential reactivity for these residues.

Table 5. *Environments of the amino groups of HEWL.*

Amino group	Area exposed to solvent [Å^2][a]	% accessibility[b]	Residues in contact[c]
Lys1, Nα	5	15.4	H-bond with OG1 Thr40
Lys1, Nε	24	61.5	H-bond with OE Glu7
Lys13	16	57	H-bond with O Leu129
Lys33	40	62	H-bond with OD1 Asn37
Lys96	21	44	H-bond with O His15
Lys97	36	48	–
Lys116	20	53	H-bond with OD1 Asn106 H-bond with O Asn113[d]

[a] Taken from ref. [46]. [b] Calculated by use of the CSU software.[47] [c] Protein data bank file 193 L.pdb. [d] Symmetrically related molecule.

Markedly different behaviour was noted when the reaction proceeded by diffusion in crystals, as no labelling was shown to occur at lysine 116. Indeed, examination of this particular residue in the crystal state showed an intramolecular hydrogen bond between its ε-NH_2 group and the δ-carbonyl of Asn106, as mentioned above, together with an intermolecular hydrogen bond with the α-carbonyl of Asn113 of a symmetrically related protein molecule (see Figure 8). All the other lysine residues are more distant from neighbouring protein molecules ($>$6 Å), thus located in the water channels. Reaction of **1** at this particular position in the tetragonal crystal state should then be sterically disfavoured.

In conclusion, MALDI-TOF MS has been shown to be a rapid and sensitive method to provide crucial semiquantitative information on the heavy metal derivatives of HEWL prepared by reaction of the novel reagent (4-ruthenocenyl) 2,6-dimethylpyrylium tetrafluoroborate in solution and by diffusion in crystals. We were able not only to evaluate the degree of incorporation of ruthenium and the nature of the bond between

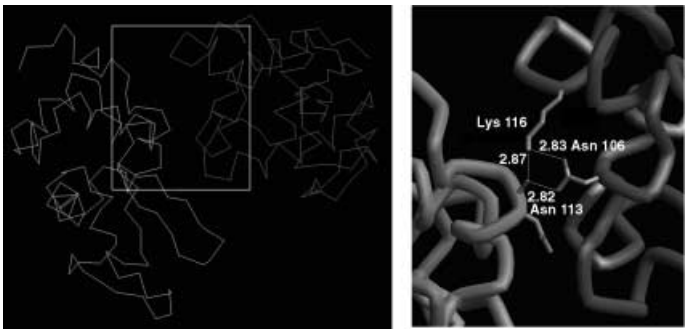

Figure 8. *X-ray tetragonal crystal structure of HEWL (PDB file 193L.pdb; two symmetrically related molecules) and an enlargement of the region of Lys116 showing the hydrogen bond network linking the ε-NH_2 group of Lys116 to the δ-carbonyl group of Asn106 and the carbonyl group of Asn113. Drawings generated by use of Swiss-PDB viewer and WebLab ViewerLite.*

the protein and the metal, but also to determine which residues provided the most favourable binding sites in solution and in the tetragonal crystal state, by combination with trypsin proteolysis. Moreover, the chemical properties of the ruthenocenyl pyrylium ion—that is, solubility, stability towards hydrolysis, ease of detection—make it an almost ideal reagent for the side chain specific heavy atom labelling of proteins. Assessment of the real usefulness of the ruthenocenyl pyrylium complex as a heavy atom reagent for phasing X-ray diffraction data is the next step to attempt.

Experimental Section

Materials: Hen egg white lysozyme (crystallized three times), TPCK-treated trypsin and dithiotreitol (DTT) were purchased from Sigma and were used without any further purification. The synthesis of 4-ruthenocenyl 2,6-dimethylpyrylium tetrafluoroborate (**1**) and 4-ruthenocenyl *N*-butyl-2,6-dimethylpyridinium tetrafluoroborate (**2**) will be described in a future paper. Buffers were prepared from double distilled grade water. Reversed-phase high performance liquid chromatography was performed on a System Gold (Beckman Coulter) machine consisting of a model 126 pump and a model 167 diode-array UV/Vis detector. UV/Vis spectra were recorded on an UV/mc^2 spectrometer (Safas).

Methods

*Reaction of HEWL with **1** in solution*: An aqueous solution of **1** (1 or 2 mM, 0.5 mL) was combined with an aqueous solution of HEWL (1 mM, 0.5 mL) buffered either with potassium phosphate (0.05 M, pH 6.8) or with HEPES (0.1 M, pH 7.5). Mixtures were left to stand in the dark at room temperature (21 $\pm$ 2 °C) from 17 h to 9 days. They were then passed through a dextran gel desalting column (bed volume = 5 mL, Pierce Chemicals) with water as eluent. The first 3 mL were collected and one part of the solution was freeze-dried on a Speedvac concentrator (Savant) to provide the samples for MALDI-TOF MS. The remaining part was kept at 4 °C for further analysis. The average number of ruthenocenyl pyridinium groups bound per protein CR was evaluated as follows. Firstly, concentration of ruthenocenyl pyridinium groups [**2**] was determined spectrophotometrically at 400 or 315 nm ($\varepsilon^2_{400} = 3950\,M^{-1}cm^{-1}$, $\varepsilon^2_{315} = 14\,500\,M^{-1}cm^{-1}$). The concentration of lysozyme [HEWL] was determined spectrophotometrically by assuming $\varepsilon^{HEWL}_{280} = 38\,000\,M^{-1}cm^{-1}$[54] after subtraction of the contribution of the organometallic group at this wavelength ($\varepsilon^2_{280} = 7800\,M^{-1}cm^{-1}$). CR was obtained by dividing [**2**] by [HEWL].

Crystal soaking experiments: HEWL crystals were grown in hanging drops, initially produced from solutions of HEWL (50 mg mL^{-1}, 4 μL) in water and of the reservoir solution (0.8 M NaCl in 50 mM sodium acetate buffered to pH 4.5, 4 μL) at room temperature (21 $\pm$ 2 °C).[55] Large, tetragonal crystals grew within one week. Some of these were transferred into drops of potassium phosphate (0.025 M, pH 6.8, 4 μL) or HEPES buffer (0.1 M, pH 7.5 containing 1 M NaCl) and were left to equilibrate for 15 min. A solution of **1** in the same buffer (1.5 mM; 4 μL) was added to the drop. Soaking proceeded from one day to three months at room temperature. Occasionally, soaked crystals were transferred into a drop of reservoir solution to remove any unbound organometallic material by back-soaking.[56]

Proteolysis of protein samples with trypsin: The ruthenocenyl pyridinium lysozyme conjugate prepared in solution was proteolysed according to the Canfield procedure.[27] Briefly, the lysozyme conjugate (570 μg, 40 nmol) dissolved in Tris·HCl buffer (0.1 M, pH 8 containing 1 mM EDTA and 6 M guanidine) was treated with DTT (10 mM) for 2 h at 25 °C and then with iodoacetic acid (50 mM) for 1 h at 25 °C in the dark. The reduced and carboxymethylated protein was dialysed in NH_4HCO_3 (50 mM, pH 8, 1 L) and the resulting suspension was treated with trypsin (1:50 *w/w*) for 4 h at 37 °C. The solution was freeze-dried on a Speedvac concentrator (Savant). The lysozyme crystals were proteolysed according to a procedure adapted from previously published work.[57] Crystals were dissolved in water (80 μL). A solution of DTT in water (0.1 M, 10 μL) was added to the resulting solutions (containing 80 to 100 μg of protein). Mixtures were incubated for 30 min at 56 °C. Cysteines were blocked by addition of iodoacetic acid (0.2 M, 10 μL) in water and incubation at room temperature for 30 min in the dark. The pH of the medium was adjusted to 8 by addition of NH_4HCO_3 (0.5 M, 10 μL). Proteolysis was performed by addition of a solution of trypsin in the proportion 1:20 (w/w) with incubation for 4 h at 37 °C. The reaction was stopped by addition of HCl (1 M, 2 μL) and the samples were freeze-dried in a Speedvac concentrator (Savant).

Reversed-phase HPLC of lysozyme conjugates: Lysozyme conjugates (20 μL or 200 μL at the semipreparative scale) were injected into a Si C4 (5 μm, 4.6 $\times$ 150 mm) 214 TP column (Vydac) equilibrated with 30% of a 0.1% TFA/MeCN mixture in 0.1% TFA/water. Species were eluted at a flow rate of 1 mL min^{-1} by application after 3 min of two successive linear gradients to 32% in 1 min then to 40% in 15 min (or 38% in 37 min at the semipreparative scale). Absorbance at 280, 315 and 400 nm were simultaneously monitored to detect unmodified protein and labelled protein adducts, respectively. For the semi-preparative scale injection, fractions (0.5 mL) were collected with an automatic fraction collector (Pharmacia). These solutions were immediately neutralised by addition of aqueous NH_3 (30 mM, 0.5 mL) and freeze-dried in the Speedvac concentrator.

Reversed-phase HPLC of tryptic peptide mixtures: Samples (20 μL) were injected into a Si C18 HD (5 μm, 3 $\times$ 250 mm) column (Macherey Nagel) equilibrated with 1% of a 0.1% TFA/MeCN mixture in 0.1% TFA/water. Species were eluted at a flow rate of 0.5 mL min^{-1} by application after 5 min of a linear gradient up to 60% in 51 min. Absorbance at 225 and 400 nm were monitored to detect unlabelled and ruthenocenyl pyridinium containing peptides, respectively. Fractions (0.5 mL) were occasionally collected, neutralised and freeze-dried as above.

MALDI-TOF MS analysis: MALDI mass spectra were recorded with a PerSeptive Biosystems Voyager Elite (Framingham, MA, USA) time-of-flight mass spectrometer. This instrument was equipped with a nitrogen laser (337 nm), delayed extraction and a reflector. It was

operated at an accelerating potential of 20 kV in both linear and reflection modes. The mass spectra shown represent an average over 256 consecutive laser shots (3 Hz repetition rate). Peptides (neurotensin, ACTH 18 – 39, apomyoglobin) were used to calibrate the mass scale through the use of the two points calibration software 3.07.1 from PerSeptive Biosystems. Matrix and calibration compounds were from Sigma and were used without further purification. The matrices—3,5-dimethoxy-4-hydroxycinnamic acid (SA) and α-cyano-4-hydroxycinnamic acid (HCCA)—were prepared as 100 mM solutions in acetonitrile/0.1% TFA (30:70; v/v). For analysis of crystals, the buffer was removed with a micropipette and 0.1% TFA (5 μL) was added for solubilization. A mixture of matrix and lysozyme or conjugate solutions (1 μL) containing approximately 10 pmol of analyte was deposited onto the sample stage and allowed to dry in air. Alternatively, tryptic peptides (10 μM, 0.5 μL) solutions and matrix solutions (0.5 μL) were mixed directly on the sample stage and, after drying, were washed three times with 0.1% TFA (5 μL). Conjugates and peptide digests of lyophilised HPLC fractions were prepared in 0.1% TFA to a final estimated concentration of 50 μM (conjugates) or approximately 10 μM (tryptic peptides); this solution (10 μL) was mixed with matrix solution (100 μL) and 1 μL deposited onto the sample stage and allowed to dry in air.

Abbreviations

CR = coupling ratio, CSU = contacts of structural units, DTT = dithiotreitol, EC = extent of conjugation, ESI-MS = electrospray ionization mass spectrometry, HCCA = α-cyano-4-hydroxycinnamic acid, HEWL = hen egg white lysozyme, MALDI-TOF MS = matrix-assisted laser desorption ionization time-of-flight mass spectrometry, MLCT = metal to ligand charge transfer, MIR = multiple isomorphous replacement, NLO = nonlinear optical, RP-HPLC = reversed-phase HPLC, SA = 3,5-dimethoxy-4-hydroxycinnamic acid, TFA = trifluoroacetic acid, TPCK = *N-p*-tosyl-L-phenylalanine chloromethyl ketone.

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Keywords: lysine residues · mass spectrometry · proteins · ruthenium · x-ray diffraction

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