Metallo-carbonyl complexes based on the $CpFe(CO)_2(\eta^1$-*N*-imidato) system as protein labelling reagents: reactivity and selectivity studies using bovine serum albumin as a model protein†

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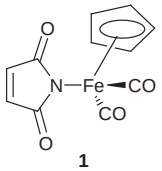
Transition metallo-carbonyl complexes are useful bioprobes to study molecular recognition processes such as ligand-receptor interactions, owing to their intense absorption bands in the mid-IR spectral range, which enable their detection at the picomole level with FT-IR spectrometers. New chemical methods for the introduction of cyclopentadienyl iron dicarbonyl fragments into proteins have been developed. Synthesis of a maleimide and of an *N*-succiminidyl ester and their reactivity with a model protein, namely bovine serum albumin (BSA), are described here. Results show that BSA can be labelled by both reagents at neutral to basic pH, and, as for the maleimide, side-chain selectivity can be tuned as a function of experimental coupling conditions.

Organo-transition metal complexes containing carbon monoxide ligands are known to display particular features in the mid-IR spectral region (2150–1800 cm^{-1}) in the form of intense and characteristic absorption bands, owing to the stretching vibration modes of the carbon—oxygen bonds. This spectroscopic property has enabled their detection by FT-IR spectroscopy down to the picomole level, even from aqueous solutions and biological systems,[1] so that this new family of non-isotopic probes proved useful in the study of biochemical association processes such as receptor-ligand, enzyme-substrate or antigen-antibody reactions.[2]

From the chemical point of view, the problem of labelling increasingly sophisticated biomolecules such as proteins soon arose. In order to address it, transition metallo-carbonyl *N*-succinimidyl esters have been prepared and successfully reacted with proteins.[3] Compounds containing this function are able to selectively acylate protein terminal and side-chain amino functions and are frequently used for the labelling of proteins with various reporter groups.[4] Other examples of organometallic labelling reagents bearing other amino-targeting functional groups, such as pyrylium salts,[5] isothiocyanate[6] or imidoester,[7] have been described.

In protein labelling there is a special interest in reagents selective for sulfhydryl groups (of cysteine residues) because these functions are less common (especially the accessible ones) so that reporter groups may be introduced at a restricted number of sites along the protein sequence.[8] As a rule, such reagents contain maleimido or iodoacetamido reactive functions.[9] We have recently reported the synthesis of the first metallo-carbonyl marker containing a maleimido function,[10] **1**, and tested its reactivity with some model aminoacids and a dipeptide.[11]



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We have found that the ethylenic bond of **1** undergoes a nucleophilic attack at neutral pH and 35 °C not only by the thiol group of the cysteine methyl ester, as generally expected from maleimides, but also by the imidazole group of the histidine methyl ester and by the dipeptide carnosine (β-alanylo-L-histidine). Moreover, in alkaline solutions, **1** also reacts with the amino group of glycine and β-alanine.

At this stage, it was then interesting to study the reactivity of **1** toward a larger polypeptide containing the three potentially reactive groups and whether this reactivity could be tuned by changing the operating conditions. We chose to react compound **1** with bovine serum albumin (BSA), which we had previously taken as a model to study the reactivity of other families of reagents. The results of this study are reported in this paper.

In addition, we were interested in the transformation of readily accessible **1** into other reagents having functional groups specific for other target functions. In this paper, we report the simple transformation of **1** into a marker containing an *N*-succinimidyl ester function, namely **3**. Its reactivity towards β-alanine and BSA was also studied.

Experimental

All reactions were carried out under an argon atmosphere. Chromatographic purifications were performed using Kieselgel 60 silica gel (Merck, 230–400 mesh ASTM). Chloroform and dichloromethane were distilled over CaH_2; other solvents were 'pure for analysis' grade and used without purification. Compound **1** was prepared according to a previously published procedure.[10] Reagent grade thioglycolic acid, NHS, DCC (1.0 M solution in dichloromethane) and β-alanine were purchased from Aldrich and used as received. NEM, TNBS, reduced glutathione and *N*-acetyl histidine were obtained from Sigma. DEPC and DTNB were obtained from Lancaster. BSA (IgG free, lipid-free, crystallised) was purchased from Serva. Phosphate (0.1 M, pH 7.0 or 7.4), borate (0.1 M, pH 8.0 or 9.0) and carbonate (0.1 M, pH 9.5) buffers were prepared

† Abbreviations used: FT-IR, Fourier transform infrared; NHS, *N*-hydroxysuccinimide; BSA, bovine serum albumin; DCC, dicyclohexycarbodiimide; NEM, *N*-ethylmaleimide; DTNB, dithiobisnitrobenzoic acid; TNBS, trinitrobenzenesulfonic acid; DEPC, diethyl pyrocarbonate; Cp, cyclopentadienyl; Fp, cyclopentadienyl iron dicarbonyl.

from demineralised water. Gel filtration chromatography was carried out on Econopac 10DG desalting columns (Biorad). ^{1}H NMR spectra were taken on a Varian Gemini 200BB (200 MHz) spectrometer. Qualitative IR spectra were taken on a Specord 75 IR apparatus whereas quantitative studies were carried out on a Bomem Michelson MB100 equipped with a liquid nitrogen-cooled InSb detector. Elemental analyses were determined by the analytical service of the Centre of Molecular and Macromolecular Studies of the Polish Academy of Sciences (Lodz). FAB spectrum was recorded on a Finnigan MAT 95 spectrometer in a glycerine matrix. UV/VIS spectra were recorded on a Safas uv/mc^2 spectrometer.

Synthesis of (η^5-cyclopentadienyl)[η^1-1-3-(carboxymethylthio)succinimidato]dicarbonyliron(II), 2

To a suspension of **1** (300 mg, 1.1 mmol) and thioglycolic acid (101 mg, 1.0 mmol) in 8 ml of water a 1 N aqueous solution of K_2CO_3 was added dropwise to obtain pH 9–10. After 2 days at room temperature, the reaction mixture was extracted several times with chloroform and then acidified with aqueous HCl to pH 2. Extraction with the same solvent, drying with Na_2SO_4 and evaporation to dryness gave a yellow oil, which crystallised from dichloromethane–ether–hexanes at −20 °C to afford **1** as a yellow solid. Yield 185 mg (48%). ^{1}H NMR (δ, $CDCl_3$): 5.07, s, 5H, Cp; 3.9, m (partly overlapped), 1H, H-3; 3.84, d (J = 14.9 Hz), 1H and 3.35, d (J = 14.9 Hz), 1H, SCH_2; 3.09, dd (J = 18.4 Hz, 9.0 Hz), 1H and 2.45, dd (J = 18.4 Hz, 3.7 Hz), 1H, succinimide CH_2. IR ($CHCl_3$, ν/cm^{-1}): 2055, 2000, Fe—CO; 1725, CO (acid); 1645, 1630, CO (imide). Anal. calcd (%) for $C_{13}H_{11}FeNO_6S$: C 42.76; H 3.04; N 3.84; S 8.78; found C 42.85; H 3.00; N 3.87; S 8.41.

Synthesis of (η^5-cyclopentadienyl)[η^1-1-3-(carbosuccinimidyloxymethylthio)succinimidato]dicarbonyliron (II), 3

2 (200 mg, 0.55 mmol) and NHS (63 mg, 0.55 mmol) were dissolved in dichloromethane (5 mL). To this solution, 0.55 ml of a 1 M solution of DCC in dichloromethane (0.55 mmol) was added and the resulting solution was magnetically stirred 24 h at room temperature. The solid formed was filtered off and the filtrate evaporated to dryness. Column chromatography afforded crude **3** as a yellow oil eluted with chloroform. Crystallisation from dichloromethane–hexanes at −20 °C afforded an analytically pure sample of **3**. Yield 197 mg (77%). ^{1}H NMR (δ, $CDCl_3$): 5.05, s, 5H, Cp; 4.31, d (J = 15.8 Hz), 1H, and 3.64, d (J = 15.8 Hz), 1H, SCH_2; 3.96, dd (J = 8.9 Hz, 4.1 Hz), 1H, H-3; 3.07, dd (J = 18.1 Hz, 8.9 Hz), 1H, H-4; 2.86, s, 4H, NHS; 2.44, dd (J = 18.1 Hz, 4.1 Hz), 1H, H-4. IR ($CHCl_3$, ν/cm^{-1}): 2055, 1995, Fe—CO; 1810, 1785, 1740 (active ester), 1645 (imide). Anal. calcd (%) for $C_{17}H_{14}FeN_2O_8S$: C 44.18; H 3.05: N 6.06; S 6.94; found C 43.81; H 3.06; N 6.06; S 6.87.

Reaction of 3 with β-alanine

3 (50 mg, 0.11 mmol) was dissolved in THF (2 mL) and a solution of β-alanine (9.5 mg, 0.11 mmol) in 4 ml of 2.5% aqueous K_2CO_3 was added. The resulting pH was ≈9. After standing for 2 h at room temperature, the reaction mixture was concentrated under reduced pressure at room temperature and extracted with chloroform. The aqueous layer was then acidified to pH ≈2 and extracted with chloroform. Drying (Na_2SO_4) and evaporation to dryness gave **4** as a yellow oil. Yield 23 mg (46%). ^{1}H NMR (δ, $CDCl_3$): 7.7 br s, 1H, COOH; 5.07, s, 5H, Cp; 4.2, very br, 1H, NH; 3.73, m (partly overlapped), 1H, H-3; 3.67, d (J = 15.8 Hz), 1H and 3.35, d (J = 15.8 Hz), 1H, SCH_2; 3.60, m, 2H, NCH_2; 3.07, dd (J = 16.5 Hz, 9.3 Hz), H-4; 2.65, broadened t, 2H, CH_2COO; 2.47, dd (J = 16.5 Hz, 3.0 Hz), 1H, H-4. IR ($CDCl_3$, ν/cm^{-1}): 2055, 2050, Fe—CO; 1740 (COOH); 1685 (amide I); 1655 (imide); 1515 (amide II). FABMS (positive ion, m/z): 447 ([M + H]$^+$, 79); 381 ([M + H − 2CO]$^+$, 18); 315 (possibly [M − 2CO − Cp]$^+$, 100).

Kinetic studies

Ten microlitres of a 1 × 10^{-2} M solution of **1** in ethanol was added to 1 mL of a 1 × 10^{-4} M solution of *N*-acetylhistidine or glutathione in phosphate buffer pH 7.4. Mixtures were incubated at 20 or 35 °C and reactions were monitored at 251 nm. The same procedure was repeated by replacing complex **1** with NEM and using ten-fold higher concentrations. Mixtures were incubated at 20 or 35 °C and reactions were monitored at 300 nm. Second-order rate constants k and half-lives were calculated from the equation $(A_\infty - A_0)/(A_\infty - A) = kct + 1$ where A_0 is the initial absorbance, A_∞ is the final absorbance and c the initial concentration of reagents.

Reaction of 1 with BSA

Procedure A. BSA (360 nmol) and **1** (4 μmol, 11 molar equiv.) were incubated in 1 mL of buffer (pH 7.4 or 9.5) containing 10% ethanol at 20 or 35 °C for 8 h. Solutions were filtered, then chromatographed on a gel filtration column with phosphate buffer as eluent. Elution was monitored spectroscopically at 280 nm in 0.5 mL fractions. Fractions containing the protein were pooled and subjected to analyses as described below.

Procedure B. BSA (300 nmol) and **1** (18 μmol, 60 molar equiv.) were incubated in 3 mL of buffer (pH 7.4 or 9.5) containing 10% ethanol at 35 °C for 3 days. Solutions were centrifuged at 4000 rpm, then gel filtered with phosphate buffer as eluent. Ten 1 mL fractions were collected and analysed spectroscopically. The first four fractions were pooled and subjected to further analyses. A control experiment was performed under the same conditions (at pH 7.4) by replacing **1** with NEM.

Reaction of 3 with BSA

To 1 mL of a 50 μM BSA solution in phosphate (pH 7.0) or borate (pH 8.0 or 9.0) buffer was added 0.1 mL of a 0.03 M solution of **3** in DMF (60 molar equiv.). The reaction mixture was stirred 1 h at room temperature, then gel filtered. Species were eluted with 10 mM ammonium acetate. Fractions were collected and chromatographic profiles were plotted from absorbances read at 2053 cm^{-1}. Fractions containing the protein were pooled and the conjugates analysed as described below.

Analysis of the conjugates

The protein concentration was measured by the Coomassie blue method.[12] The concentration of cyclopentadienyl iron dicarbonyl (Fp) groups was measured by IR spectroscopy of the carbonyl ligand band at 2050 cm^{-1} ($k = A/c = 12$) as previously described.[3b] The concentration of free thiols was measured by the reaction with DTBN and quantitation of the released TNB^{2-} at 412 nm (ε = 13,700).[13] The concentration of free imidazoles was measured by reaction with DEPC followed by difference UV spectrometry at 242 nm (ε = 3,200).[14] The concentration of amino groups was measured by the TNBS method.[15]

Results and Discussion

Kinetic studies of the reaction of 1 with model molecules

The reaction of maleimide **1** with glutathione and *N*-acetylhistidine was monitored spectroscopically at room temperature and 35 °C. Second-order rate constants were calculated and compared to those of NEM with the same sub-

Table 1 Kinetic studies of the alkylation of *N*-acetyl histidine and glutathione with **1** and NEM at pH 7.4

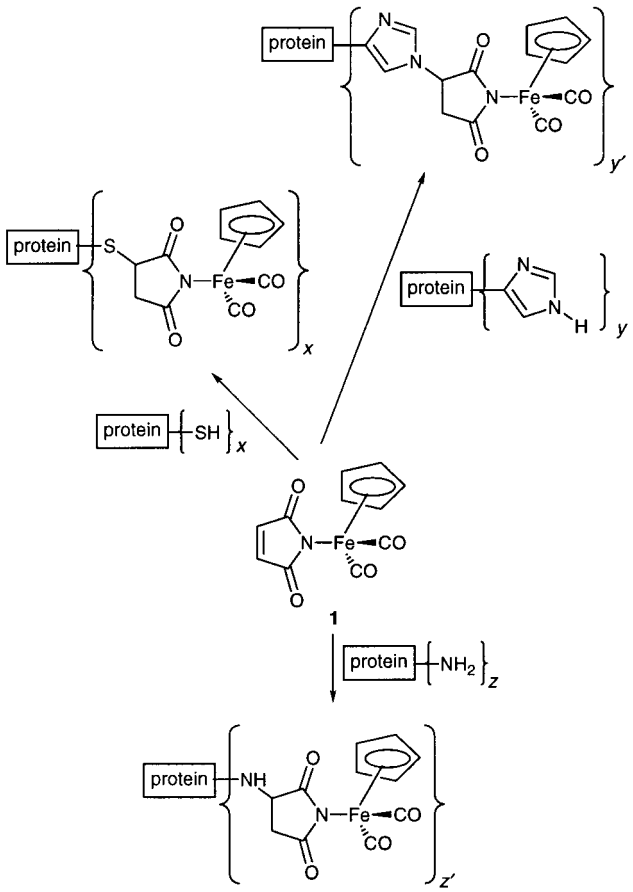
	1				NEM			
	N-Acetyl histidine[a]		Glutathione[a]		*N*-Acetyl histidine[b]		Glutathione[a]	
T/°C	*k*/ M^{-1} min^{-1}	half-life/ min	*k*/ M^{-1} min^{-1}	half-life/ min	*k*/ M^{-1} min^{-1}	half-life/ min	*k*/ M^{-1} min^{-1}	half-life/ min
20	No reaction	—	382	26	No reaction	—	9523	1
35	84	119	370	27	5	205	nd	nd

[a] Concentration of reagents equal to 1×10^{-4} M. [b] Concentration of reagents equal to 1×10^{-3} M.

strates. Data are collected in Table 1. At room temperature (after 24 h), no reaction of either **1** or NEM was observed when the substrate was *N*-acetylhistidine. On the other hand, the reaction with glutathione proceeded readily with the expected formation of the *S*-alkylation compound as evidenced by comparison with an authentic sample of labelled glutathione. At 20 °C, the reaction rate was 25 times higher with NEM than with maleimide **1**, in accordance with the relative deactivation of the ethylenic bond of the maleimide ring by the presence of the Fp moiety. At 35 °C, both maleimides reacted with *N*-acetylhistidine and this time **1** reacted faster than NEM but the reaction rate was lower than with glutathione.

Labelling of BSA with 1

On account of the observed reactivity of complex **1** towards amino acids and amino esters (cysteine methyl ester, histidine methyl ester, glycine and β-alanine), it appeared interesting to study the behaviour of **1** toward a protein possessing simultaneously sulfhydryl, imidazole and amino functions (Scheme 1). Bovine serum albumin (BSA) was chosen as a model because it is an inexpensive protein frequently used for derivatisation studies with various molecules (haptens, reporter groups, *etc.*). BSA is a 67 kDa globular protein consisting of 582 amino acids, including 59 lysines (30–40 lysines are believed to be accessible to coupling reagents), 17 histidines and theoretically one cysteine.[16]



Scheme 1 Possible adducts from the reaction of maleimide **1** with BSA

Reaction of BSA with a ten-fold molar excess of **1** was performed at pH 7.4 and 9.5 and at room temperature or 35 °C. Protein conjugates were separated from excess reagent by gel filtration chromatography. A typical chromatogram obtained by plotting the absorbance of each fraction at 280 nm, 360 nm and 2050 cm^{-1} *versus* the elution volume is displayed Fig. 1. Each trace displays two well-separated peaks at $v = 2$ and 5 ml, readily assigned to the protein-bound and the free Fp groups, respectively. In each case, the fractions containing the protein were pooled and the samples were submitted to several analyses.

First, quantitation of the conjugate sulfhydryl groups was done by Ellman's method.[13] Second, quantitation of the number of Fp groups (*i.e.*, the Fp : protein molar ratio) was achieved by IR absorption spectroscopy of the two characteristic ν_{CO} bands at 2050 and 2000 cm^{-1}, using an original sampling method compatible with the analysis of aqueous solutions. This method consists in depositing a small volume of conjugate solution (typically 10 μl) on a nitrocellulose membrane and evaporating the solvent by simple air drying. The transmission spectrum of the membrane is recorded and subtracted from the spectrum of an untreated membrane in the 1800–2200 cm^{-1} spectral region. Calibration is performed in the same manner with solutions of complex **1** in water–ethanol. The resulting curve is shown in Fig. 2 along with the original spectra.

The conjugate characteristics are gathered in Table 2. They call for the following comments. Whatever the reaction conditions applied, the number of surviving sulfhydryl functions was close to nil. Surprisingly, even native BSA had a number of thiol groups lower than theoretically expected. This is in accordance with the fact that commercial preparations of BSA are heterogeneous and contain molecules with blocked cysteine-34.[16] Secondly, the Fp : protein ratio increased with

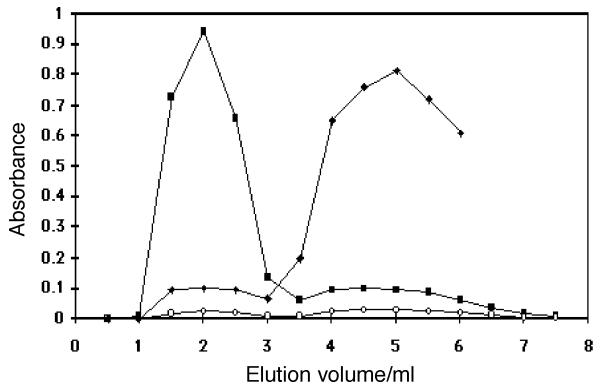


Fig. 1 Gel filtration chromatography of the reaction of BSA with **1** at pH 7.4 and 20 °C. Absorbance at (■) 280 nm, (○) 360 nm, (◆) 2050 cm^{-1}

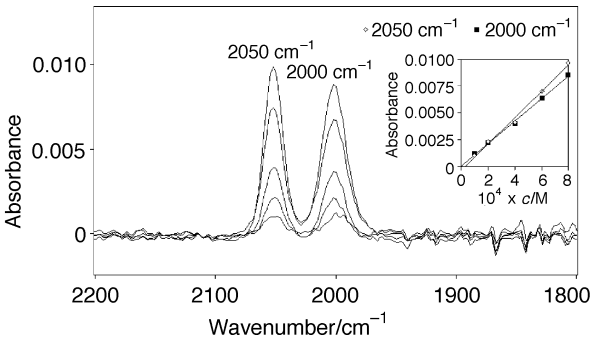


Fig. 2 IR spectra of variable quantities of **1** in the 1800–2200 cm^{-1} region. For sampling methods see text. Inset: calibration curve. Linear regression at 2050 cm^{-1} (◇): $y = 12.17x - 0.0003$, $r^2 = 0.994$. Linear regression at 2000 cm^{-1} (■): $y = 10.46x + 0.0001$, $r^2 = 0.997$

pH and temperature and except for the reaction performed at neutral pH and room temperature, the Fp : protein ratio was always greater than the initial number of thiols. Therefore, it can be concluded that specific labelling of BSA thiols is achieved at neutral pH and room temperature, whereas other nucleophilic side chains are involved to a low extent in the labelling of BSA at 35 °C or basic pH. Considering the overall very low coupling ratios measured under these conditions, it was impossible to determine which side chains were involved from this first series of experiments.

A second series was then carried out with a larger quantity of reagent (60 molar equiv.) and a longer reaction time (3 days). Resulting conjugate samples were assayed for their Fp, sulfhydryl, imidazole and amino groups content with standard group-specific reagents (Scheme 2). Results are displayed in Table 3. It was found that at pH 7.4, all the sulfhydryl and some imidazole groups were labelled by complex **1** and the same behaviour was found for the popular thiol blocking agent NEM. At pH 9.5, a concomitant high labelling of amino groups was observed, promoted by partial deprotonation of this function. However, in this latter case, the total number of blocked side chains (0.5 thiols + 2.5 imidazoles + 31 amino groups = 34 functions) slightly exceeded the number of Fp groups (27.7). This could be due to partial decomplexation of the Fp complex at basic pH and indeed, some brown precipitate was observed at the end of the reaction.

This relative non-specificity of the reaction of maleimides towards various protein nucleophiles has been reported several times, when applying particular coupling conditions (high concentration of maleimide, high pH).[17] We show here that temperature has also an effect by allowing concomitant reaction of cysteines and imidazoles. These results are also in full agreement with those previously obtained with the model aminoacids.[11]

Table 2 Conjugation of BSA with **1** (8 h at 20 or 35 °C). Analysis of the conjugates

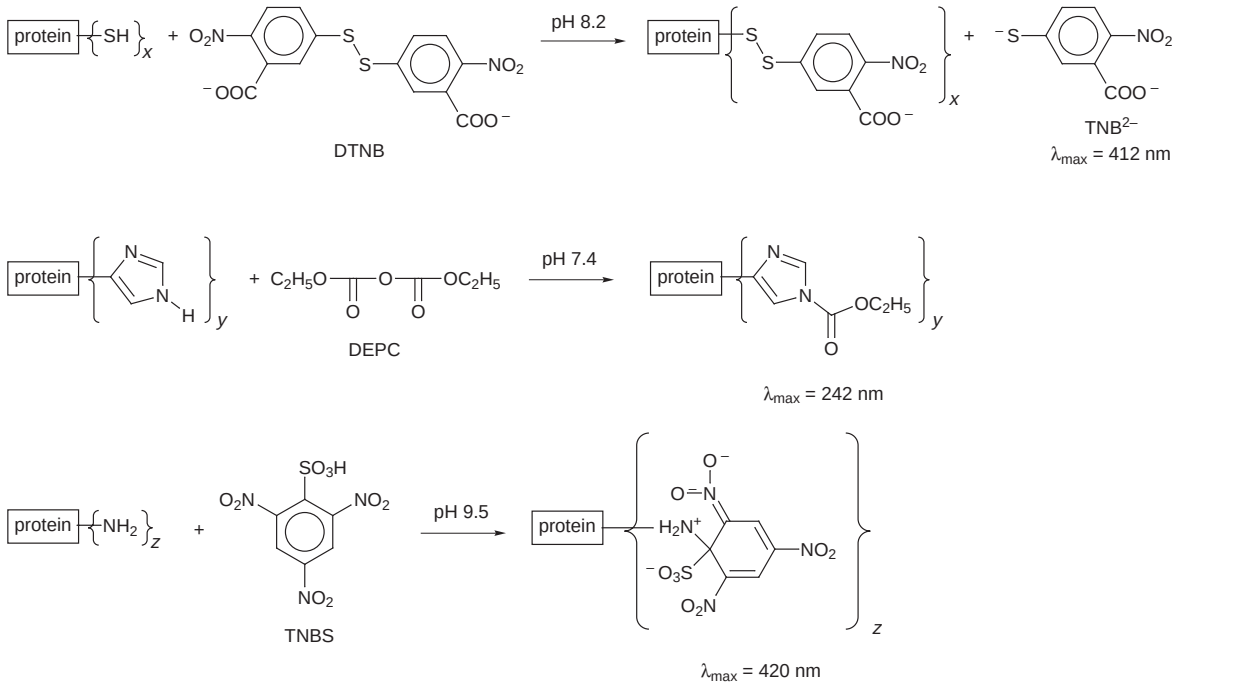
Reagent	none	**1**		
T/°C	—	20	35	20
Reaction medium pH	—	7.4	7.4	9.5
Fp : protein molar ratio	0	0.32	0.65	0.82
Number of free thiols	0.38[a]	0.03	0.02	0.05

[a] Measured by Ellman's method.[13]

Table 3 Conjugation of BSA with **1** or NEM (3 days at 35 °C). Analysis of the conjugates

Reagent	none	NEM	**1**	
Reaction medium pH	—	7.4	7.4	9.5
Fp : protein molar ratio	0	0	2.9	27.7
Number of thiol groups	0.5[a]	0	0	0
Number of imidazole groups	17[b]	15.0 ± 0.5	14.8 ± 0.5	14.4 ± 0.5
Number of amino groups	60[b]	60	63	28.8

[a] Measured by Ellman's method.[13] [b] Taken from ref. 16.



Scheme 2 Scheme of the spectrophotometric assays of protein sulfhydryl, imidazole and amino groups

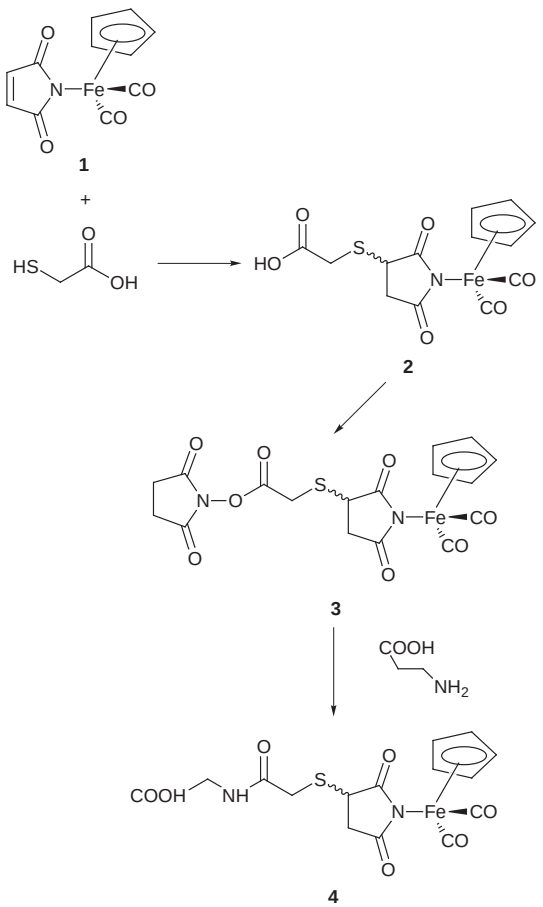
Synthesis of 3 and reaction with β-alanine

We found that **1** readily reacted with thioglycolic acid to afford the addition product of the thiol group to the ethylenic bond of the maleimide moiety, **2** (Scheme 3). This product was transformed into the corresponding *N*-succinimidyl active ester **3** by routine treatment with NHS and DCC in dichloromethane. Both complexes were fully characterised by elemental analysis and spectroscopic methods. Before starting experiments with BSA, the reactivity of **3** toward a primary amine was tested using β-alanine as a model substrate, in THF-H_2O solution at pH 9. We isolated complex **4** and characterised it by ^{1}H NMR, IR and FAB-MS.

Labelling of BSA with 3

Reactivity of the new *N*-succinimidyl ester **3** with BSA was explored. Conjugation was performed at pH 7, 8 and 9 in the presence of 60 molar equivalents of **3**. After purification of the conjugates by gel filtration chromatography, the final Fp : protein molar ratio was measured by IR spectroscopy combined with a colorimetric protein assay as above. Results are displayed in Table 4. First, a significant pH dependence of the Fp : protein ratio is observed. This behaviour is quite classical and can be related to the increasing deprotonation of the protein amino groups that thus become available for reaction as the pH increases. This trend had been previously noticed by ourselves when testing the reactivity of a rhenium carbonyl *N*-succinimidyl ester[3b] and by others in the radio-labelling of antibodies with an iodinated *N*-succinimidyl ester.[18]

Interestingly, the acylation yield was quite acceptable at neutral pH, contrasting with the results obtained in the direct labelling of BSA with complex **1**. This emphasises again the large difference in selectivity between *N*-succinimidyl esters and maleimides.



Scheme 3 Synthesis of **3** and its reaction with β-alanine

Table 4 Conjugation of BSA with **3** (1 h at room temperature). Analysis of the conjugates

Reaction medium pH	[Fp]/μM[a]	$[P]_{fin}$/μM[b]	Fp : protein molar ratio[c]	Coupling yield[d]/%
7.0	349	47	7.4	12
8.0	476	45	10.6	18
9.0	476	40	12.0	20

[a] Final protein concentration measured by the Coomassie blue method;[12] initial concentration was 50 μM. [b] Final Fp concentration measured by IR spectroscopy; the initial concentration of **3** was 3 mM (60 molar equiv.). [c] Fp : protein molar ratio = $[Fp]_{fin}/[P]_{fin}$. [d] Coupling yield = $([Fp]/[P]_{fin})/([Fp]_{init}[P]_{init}) \times 100$.

Coupling yields with complex **3** are lower than those measured for the rhenium carbonyl *N*-succinimidyl ester mentioned above and for which we had found a label-to-protein ratio of 20 at pH 9.0, under the same experimental conditions. This lower acylation yield could be explained by the high tendency of **3** to undergo a competitive hydrolysis in aqueous medium. This hypothesis was confirmed when trying to measure the hydrolysis rate of compound **3** in water by UV spectroscopy. It appeared that hydrolysis was complete within 1 min after dilution of a fresh DMF solution of **3** in phosphate buffer (data not shown). This relative instability of ester **3** in aqueous medium could be due to the electron-withdrawing effect of the sulphur atom in the α-position of the ester group. This effect is clearly present for methylthioacetic acid, which is an order of magnitude more acidic than acetic acid (pK_a = 3.72 *vs.* 4.75). The electrophilic character of the carboxyl group carbon would then be increased, hence its enhanced reactivity with nucleophiles.

Conclusions

Reactivity of a transition metallo-carbonyl maleimide towards a model protein was explored as a function of pH. It appears that the nature of the side chains involved in the reaction markedly depends on the experimental conditions. Indeed, a specific and complete labelling of free cysteines was achieved at neutral pH and room temperature, whereas some of the histidine residues were simultaneously labelled at 35 °C. At basic pH, an additional reaction of some of the protein amino functions (mainly belonging to the lysine residues) was also established. *N*-Ethylmaleimide reactivity was also found to be identical to that of **1** and demonstrates that maleimides are not always sulfhydryl-specific reagents as widely believed by protein chemists.

Compound **1** could be easily transformed into the *N*-succinimidyl ester **3**. Its reactivity with BSA as a function of pH showed that the coupling yield followed a classical pH-dependence rule, consecutive to the increasing deprotonation of primary amino groups as the pH increases.

Both complexes **1** and **3** are good protein labelling agents and one or the other can be chosen, within particular experimental conditions, as to whether restricted or more extensive labelling ratios are wanted.

Acknowledgements

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References

1 M. Salmain, A. Vessières, G. Jaouen and I. S. Butler, *Anal. Chem.*, 1991, **63**, 2323.
2 G. Jaouen, A. Vessières and I. S. Butler, *Acc. Chem. Res.*, 1993, **26**, 361.

3 (a) A. Varenne, M. Salmain, C. Brisson and G. Jaouen, *Bioconjugate Chem.*, 1992, **3**, 471. (b) M. Salman, M. Gunn, A. Gorfti, S. Top and G. Jaouen, *Bioconjugate Chem.*, 1993, **4**, 425. (c) A. Gorfti, M. Salmain, G. Jaouen, M. J. McGlinchey, A. Bennouna and A. Mousser, *Organometallics*, 1996, **15**, 142.
4 S. S. Wong, *Chemistry of Protein Conjugation and Cross-linking*, CRC Press, Boca Raton, Florida, 1991.
5 M. Salmain, K. L. Malisza, S. Top, G. Jaouen, M.-C. Sénéchal-Tocquer, D. Sénéchal and B. Caro, *Bioconjugate Chem.*, 1994, **5**, 655.
6 A. Kazimierczak, J. Zakrzewski, M. Salmain and G. Jaouen, *Bioconjugate Chem.*, 1997, **8**, 489.
7 S. Blanalt, M. Salmain, B. Malézieux and G. Jaouen, *Tetrahedron Lett.*, 1996, **37**, 6561.
8 A. Terpetschnig, J. D. Dattelbaum, H. Szmacinski and J. R. Lakowicz, *Anal. Biochem.*, 1997, **251**, 241.
9 (a) M. Brinkley, *Bioconjugate Chem.*, 1992, **3**, 2. (b) R. Lundblad, in *Protein Labfax*, ed. N. C. Price, Bios Scientific Publishers and Academic Press, San Diego, 1996, pp. 287–298.
10 B. Rudolf and J. Zakrzewski, *Tetrahedron Lett.*, 1994, **35**, 9611.
11 B. Rudolf and J. Zakrzewski, *J. Organomet. Chem.*, 1996, **522**, 313.
12 M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248.
13 G. L. Ellman, *Arch. Biochem. Biophys.*, 1959, **74**, 443.
14 E. W. Miles, *Methods Enzymol.*, 1977, **47**, 431.
15 S. L. Snyder and P. Z. Sobocinski, *Anal. Biochem.*, 1975, **64**, 284.
16 T. Peters Jr., *Adv. Protein Chem.*, 1985, **37**, 161.
17 (a) C. F. Brewer and J. P. Riehm, *Anal. Biochem.*, 1967, **18**, 248. (b) T. E. Creighton, in *Protein Structure*, ed. T. E. Creighton, IRL Press, Oxford, 1989, pp. 155–167. (c) A. Papini, S. Rudolf, G. Siglmüller, H.-J. Musiol, W. Görhing and L. Moroder, *Int. J. Pept. Protein Res.*, 1992, **39**, 348.
18 G. Vaidyanathan and M. R. Zalutsky, *Bioconjugate Chem.*, 1990, **1**, 269.

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