FTIR Detection of an Enzyme-bound Organometallic Carbonyl Probe in the Presence of the Unbound Probe Molecule

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High-frequency shoulders on ν_s(CO) and ν_{as}(CO) bands in the FTIR spectra of an organometallic probe derived from tricarbonyl(1–4-η-5-pyridiniocyclohexa-1,3-diene)iron hexafluorophosphate have been observed on incubation with α–chymotrypsin and were assigned to an iron complex covalently bonded to enzyme-NH_2 groups; curve-fitting analysis of the spectra enabled the bound complex to be detected even in the presence of an excess of the unbound probe and its FTIR spectrum to be calculated and compared with spectral data arising from similar interactions between the tricarbonyliron moiety and 12 amino acids and polylysine.

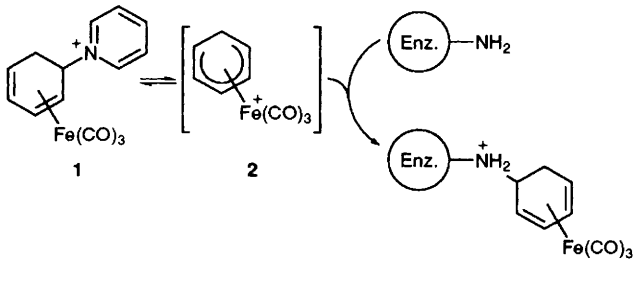
Organometallic carbonyl complexes are novel probes for the study of protein receptors.[1] Recent developments include the demonstration of the CMIA (carbonylmetalloimmunoassay) method by the Jaouen group[2] and the selective covalent attachment of tricarbonyliron complexes to amino acid side-chain functionality in proteins.[3] We have examined in detail the use of FTIR spectroscopy to reveal the effects of changes in the environment of organometallic carbonyl probe groups.[4,5] Both solvation[4] and pH[5] effects have been determined by FTIR. We now report the detection of enzyme-bound tricarbonyliron complexes in the presence of an excess of the unbound probe molecule by FTIR using curve-fitting analysis of the spectra. This method obviates the need to wash away any excess organometallic reagents before spectroscopic analysis (in contrast to the CMIA procedure), opening the way for *in situ* studies of proteins at low concentrations, exploiting the sensitivity[2] of FTIR detection. It also offers possibilities for the read-out of information concerning the immediate environment of the protein-bound probe.

Unlike recently reported studies,[3] which employed η^5-dienyl complexes directly, we have used an η^4 precursor **1**[6] bearing a labile substituent (a pyridine leaving group). By selecting a suitable leaving group, the rate (and pH sensitivity) of the generation of the reactive cationic dienyl complex **2** can be controlled. In the case of **1**, we report evidence indicating that ease of dissociation of the pyridinium salt leads to the covalent attachment of the probe to surface amino groups on a protein analyte.†

An initial series of experiments examined transfer of the tricarbonyl(cyclohexadiene)iron moiety to the enzyme α-chymotrypsin at pH 7.5. The FTIR spectrum (InSb detector; 0.5 cm^{-1} resolution) of **1** (0.08 ml methanol solution of **1** added to 1 ml 0.2 mol dm^{-3} potassium phosphate buffer; $[\mathbf{1}]_{final} = 1.65 \times 10^{-3}$ mol dm^{-3}) in the 2100–1900 cm^{-1} region, showed the expected[6] two bands: one at 2051.3 cm^{-1} assigned to the ν_s(CO) stretching mode, and an overlapped band system centred at 1979.5 cm^{-1} assigned to the non-degenerate ν_{as}(CO) stretching modes. The latter could be decomposed using curve-fitting analysis into the two component bands at 1981.8 and 1974.9 cm^{-1}. The spectrum showed no change with time. In the presence of α–chymotrypsin ($1.81–4.46 \times 10^{-3}$ mol dm^{-3}), high-frequency shoulders appeared on both bands. These signals slowly increased in intensity with time [Figs. 1(*a*)–(*f*)], indicating the formation of a covalent bond between α-chymotrypsin and the organometallic carbonyl probe.

Despite the presence of an excess of unbound **1**, the positions of the new IR bands (2058.1 and 1991.5 cm^{-1}) could be determined accurately using curve-fitting analysis [Fig. 1(*g*)].‡ These vibrational modes occur at higher wavenumber than those reported[3] for protein or peptide adducts bound to the organometallic carbonyl unit by His (imidazole) or Cys (thiol) side-chain groups. With increasing concentrations of α–chymotrypsin, we observed an increase in reaction rate and a decrease in the induction period of the reaction. This observation supports the view that the new spectral features were due to covalent interaction between the metal carbonyl moiety and the enzyme, not simple association of intact **1**.

To identify the binding sites for the organometallic carbonyl probe on the chymotrypsin molecule, a comparison was made with spectra obtained by reaction of **1** with an excess of each of a range of amino acids, under the same buffer conditions used for α–chymotrypsin. With glycine, signals corresponding to both excess **1** and a tricarbonyl(glycinocyclohexadiene)iron adduct were observed, the latter appearing at 2058.5 and 1991.8 cm^{-1}, in excellent agreement with those observed with the enzyme. Since glycine has no side-chain, **1** can react with the $-NH_3^+$ or $-CO_2^-$ groups. These alternatives were distinguished by measuring the FTIR spectrum of **1** after addition of either ethylamine·HCl or sodium propanoate.



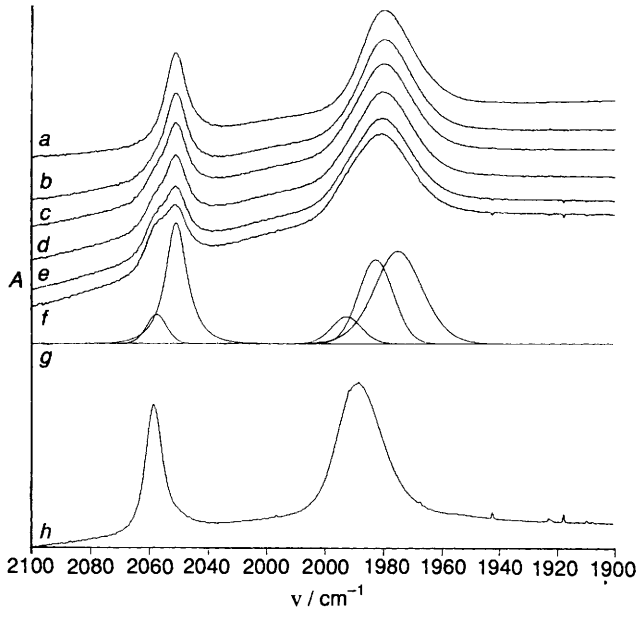


Fig. 1 (*a*)–(*f*) FTIR spectra of **1** in pH 7.5 buffer solution containing α–chymotrypsin recorded at intervals over 3 h. (*g*) Component bands resulting from decomposition of spectrum *c* using a scale expansion of 2. (*h*) FTIR spectrum of $[Fe(CO)_3(C_6H_7NH_2Pr^i)](PF_6)$ in buffer.

Table 1 FTIR data from products of reaction of **1** with protein and peptide analytes, and with model compounds

Analyte/model compound	Reactive functional group[a]	ν_s(CO)/ cm^{-1}	ν_{as}(CO)[b]/ cm^{-1}	Ref.
α-Chymotrypsin	$-NH_2$	2058.1	1991.5	
Polylysine	$-NH_2$	2058.2	1993.6	
Lysozyme	$-C_3N_2H_3$	2050	1978[c]	3
p-Glu-His-Pro-NH_2	$-C_3N_2H_3$	2050	1980[c]	3
Glutathione	–SH	2050	1966[d]	3
Glycine	$-NH_2$	2058.5	1991.8	
Alanine	$-NH_2$	2058.6	1994.0	
Isoleucine	$-NH_2$	2058.7	1994.7	
Serine	$-NH_2$	2058.6	1990.7	
Aspartic acid	$-NH_2$	2058.5	1993.7	
	$-CO_2^-$	2042.8	[e]	
Glutamic acid	$-NH_2$	2058.4	1989.4	
	$-CO_2^-$	2043.6	[e]	
N-Ac-glutamic acid	$-CO_2^-$	2045	1973[d]	3
Asparagine	$-NH_2$	2059.2	1991.1	
Glutamine	$-NH_2$	2058.6	1990.6	
Lysine	$-NH_2$	2059.2	1991.9	
Histidine	$-NH_2$	2057.1	1990.6	
N-Ac-histidine	$-C_3N_2H_3$	2056	1982[c]	3
Arginine	$-NH_2$	2058.9	1991.5	
	[f]	2042.6	[e]	
Cysteine	–SH	2038.6	1960.8	
N-Ac-cysteine	–SH	2055	1974[d]	3
$EtNH_2$·HCl	$-NH_2$	2058.4	1990.8	
$(EtCO_2)Na$	$-CO_2^-$	2044.8	1968.5	

[a] In our studies, unprotected amino acids were used; reaction does not always occur at the side chain functionality. [b] Central frequency for envelope of two overlapping bands. [c] Spectrum recorded in D_2O. [d] Nujol mull. Note that these data are not necessarily comparable with the solution wavenumbers. [e] ν_{as}(CO) could not be accurately determined (see text). [f] Unassigned.

New bands were detected at 2058.4 and 1990.8 cm^{-1} after addition of ethylamine·HCl to a solution of **1**, whereas when the experiment was performed with sodium propanoate lower-frequency bands at 2044.8 and 1968.5 cm^{-1} were observed. From this result, it was clear that it was the glycine amino group that takes part in the formation of the covalent adduct. To confirm this, the isopropylamine adduct of **2**, $[Fe(1\text{–}4\text{-}\eta\text{-}5\text{-}C_6H_7NH_2Pr^i)(CO)_3](PF_6)$, was synthesized and characterised (IR, NMR, microanalysis, FAB–MS). This compound had spectral data (2058.5 and 1991.4 cm^{-1}) very similar to those from the glycine adduct, and also those from the organometallic carbonyl adduct of α–chymotrypsin [Fig. 1(*h*)], indicating that the organoironcarbonyl moiety is covalently bonded to amino groups on the enzyme molecule.

With most of the other amino acids studied, although side-chain functionality is now present, similar new features were obtained as shoulders on the bands originating from the excess of **1**, and the wavenumbers (2059.2–2058.3 cm^{-1} and 1994.7–1990.6 cm^{-1}) were again determined by curve-fitting analysis. With histidine, ν_s(CO) for the adduct is found at 2057.1 cm^{-1}, slightly higher than the value found by Carver *et al.*[3] (2056 cm^{-1}) for reaction at the imidazole side chain. With cysteine, new bands at lower frequency were observed, at 2038.6 and 1960.8 cm^{-1}. The wavenumber data reported[3] for the isolated cysteine adduct of **2** were obtained from a Nujol mull, and so are not directly comparable with solution spectra. With aspartic acid, glutamic acid and arginine, in addition to the higher-wavenumber bands, weaker lower-wavenumber shoulders were also observed. It proved possible to locate this third component of the ν_s(CO) envelope by curve-fitting (Table 1). These lower-wavenumber bands result from reaction of **1** with side-chain functional groups.

In the α-chymotrypsin molecule,[7] 16 amino groups (14 lysine and 2 *N*-terminal) and 12 carboxylate groups (aspartate and glutamate) are accessible on the surface of the enzyme molecule. Of the two histidine residues in the molecule, one is in the active site and the other is not accessible at the surface. We do not see any evidence for reaction of **1** with the carboxylate groups; instead, selective reaction with the surface amino groups is obtained resulting in the observed high-frequency IR signals. In contrast to Carver *et al.*,[3] we observe reaction of the organoiron moiety with amino residues. This is presumably a consequence of the higher pH used in our experiments, at which a small proportion of the $-NH_3^+$ groups are deprotonated to strongly nucleophilic amino groups, allowing reaction to occur.

Our results have demonstrated the principle that a protein-bound organometallic carbonyl probe molecule can be detected and the binding site characterised, even in the presence of an excess of the unbound probe molecule. This finding is important in the design of such organometallic carbonyl probes for the development of the CMIA method, in cases where separation of protein-bound and free organometallic complexes before spectroscopic measurement is inconvenient or impractical. Binding at amine functionality can be distinguished from binding at imidazole and thiol groups in histidine and cysteine residues. Future work will focus on the use of curve-fitting analysis to determine ν(CO) wavenumbers of organometallic carbonyl-protein adducts in order to identify the site of attachment of the organometallic unit, and, by means of our PCA methods,[4] to explore the environment and properties (*e.g.* local pH[5]) of the binding site. An application of this method, employing organometallic flavonoid derivatives to study induction of *Rhizobium* nodulation gene expression in the early stages of root nodulation for nitrogen fixation in legumes[8] by delivery of organometallic probes to the active site of the suspected *Rhizobium* control protein NodD, is in progress.

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Footnotes

† Our experiments were performed before the publication of results defining the direct reaction of **2** with amino acids. Data reported in ref. 3, however, are in agreement with our interpretation of results obtained using reagent **1**.

‡ The antisymmetric band for the new species was modelled using a single band; curve-fitting the observed band envelope with four components did not always yield reliable results.

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