hibitor molecules. A careful kinetic control of the reaction should thus allow one to preferentially modify the active site residues.

As a first approach, we studied the photooxidation of α-chymotrypsin (EC 3.4.4.5) sensitized by N-acetyl-3-nitrotyrosine. We found that this compound, in slightly alkaline solutions, mediates the photooxidation of the cysteinyl, methionyl, histidyl, tyrosyl, and tryptophyl residues; moreover, when present in the molar ratio 2:1, it shows competitive inhibition of the hydrolysis of Bz-Tyr-OEt[6] by α-chymotrypsin with k_i = 1.03 m*M*.[7]

In a typical experiment, 1 μmol of α-chymotrypsin, added with 2 μmol of N-acetyl-3-nitrotyrosine in 100 ml of 0.1 *M* phosphate buffer, pH 7.8, was exposed to the light of a 1000-W high-pressure mercury lamp. The irradiation apparatus was the same as previously described.[4] After 5-min irradiation at 20°, the inhibitor and buffer were removed by gel filtration on a column of Sephadex G-25 equilibrated with 0.1 *M* acetic acid. Chromatographic analysis[8] of the recovered protein showed that over 90% of the irradiated sample was eluted as one peak. The product from the peak was isolated; it was devoid of lytic activity toward Bz-Tyr-OEt and contained one photooxidized methionyl and histidyl residue per protein molecule (see Table I).

Table I. Amino Acid Analyses of α-Chymotrypsin[a]

Amino acid	Theory	Native	Irradiated
Histidine	2	2.0	0.8
Tyrosine	4	3.8	3.8
Tryptophan[b]	8	7.8	7.8
Methionine[c]	2	1.8	1.0
Methionine sulfoxide[c]	0	0.0	0.9

[a] The amino acid content was evaluated on a Carlo Erba 3A27 amino acid analyzer after hydrolysis for 22 hr at 110° in 6 *N* HCl within evacuated sealed vials. The table includes only the amino acids potentially susceptible of photooxidation.[1,4] No change was detected in the content of the other residues. The values in the table denote number of residues per molecule. [b] Determined on the intact protein by the spectrophotometric method of T. W. Goodwin and R. A. Morton, *Biochem. J.*, **40**, 268 (1946). [c] Determined on the Carlo Erba analyzer after alkaline hydrolysis.[4]

The modified methionine was identified by allowing the irradiated protein to react with cyanogen bromide.[9] Amino end-group analysis of the reaction product according to Sanger and Tuppy[10] revealed the presence of 1.77 mol of DNP-isoleucine (accounting for the isoleucine following methionine-180 and the N-terminal isoleucine of the A chain of α-chymotrypsin) per mole of protein; on the contrary, no trace was detected of DNP-glycine, which follows methionine-192 in the amino acid sequence. Since CNBr does not attack the methionine sulfoxide residues,[9] it appears that methionine-192 is the selectively modified methionine.

(6) Abbreviations used: Bz-Tyr-OEt, N-benzoyltyrosine ethyl ester; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

(7) The concentration of chymotrypsin was evaluated spectrophotometrically on the basis of ε being 5×10^4 M^{-1} cm^{-1} at 282 nm. The enzymic activity against Bz-Tyr-OEt was assayed according to Hummel (B. C. W. Hummel, *Can. J. Biochem. Physiol.*, 37, 1393 (1959)). Inhibition studies were carried out at pH 7.8 (0.08 *M* Tris buffer) using 0.5 m*M* Bz-Tyr-OEt.

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In order to identify the photooxidized histidine, α-chymotrypsin was treated with TPCK as described by Schoellman and Shaw.[11] Whereas the unirradiated protein gave a clear reaction incorporating 0.9 mol of TPCK per mole of protein, the photooxidized sample appeared to give no reaction at all. Now, Schoellman and Shaw state that in native α-chymotrypsin TPCK specifically reacts with the imidazole ring of histidine-57. Therefore, our data may suggest that histidine-57 is the uniquely photooxidized histidine.

The conclusion that methionine-192 and histidine-57 are located at the active site of α-chymotrypsin is supported by the X-ray structure of the enzyme[12] as well as by several previous studies.[13] On the other hand, it is remarkable that no tryptophan was photooxidized under our conditions. Actually, chemical modification[14] and kinetic[15] studies suggested that one tryptophyl residue is close to the active site of α-chymotrypsin. However, Galley and Stryer[16] found no phosphorescence quenching by tryptophan after labeling of the active serine residue with a potential triplet donor. Our results corroborate the conclusion, drawn by these authors, that no tryptophan is involved in the catalytic region of the enzyme. This is also suggested by the X-ray structure.

It is hoped that similar reliable data are obtained by irradiation of other complexes between inhibitors and enzymes, the tertiary structure of whose active sites has been elucidated by independent approaches. Consequently, the photooxidative method described could be used to map the topography of enzymic active sites. Since a large variety of inhibitors is usually available for any given enzyme, it may be possible to further refine this procedure by mapping the spatial orientation of the photooxidizable residues through the use of sensitizers of different steric or chemical structure, bulkiness, and polarity. Studies concerning such applications are in progress in our laboratory.

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Nuclear Magnetic Resonance Contact Shifts of Some Binuclear Iron(III) Phenanthroline Complexes

Sir:

Contact shift studies of high-spin iron(III) complexes have in general been sparse owing to the fact that extremely broad signals are observed.[1] However, there is a group of oxo-bridged binuclear iron(III)

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complexes[2-7] having subnormal magnetic moments, which should be amenable to nmr study because exchange coupling in these complexes can significantly decrease the proton nmr line widths.[8] Furthermore there will be no complications due to pseudocontact shifts, and this will greatly simplify the interpretation of their observed isotropic shifts. Of the octahedrally coordinated complexes studied to date by contact shift nmr, only those of nickel(II) have this very desirable feature.

We have begun an investigation of the nmr contact shifts of some of these complexes and in particular wish to report our preliminary results for the complexes $[Fe_2L_4O]Cl_4 \cdot nH_2O$ where L is 1,10-phenanthroline (phen) or the symmetrically substituted dimethylphenanthrolines, 4,7-dimethylphenanthroline (4,7-dmp) and 5,6-dimethyl-1,10-phenanthroline (5,6-dmp). Recent experimental evidence including infrared[4] and conductance[6] data has shown that these complexes contain an oxo bridge and two coordinated chlorides and not two hydroxy bridges as was once believed. Furthermore magnetic measurements[4,6] and Mössbauer spectra[5] suggest that the spin states of the individual iron atoms are $S = {}^5/_2$, and that antiferromagnetic exchange coupling with $J \cong 100$ cm^{-1} and $g = 2.0$ can explain the variation of χ_m with T and the subnormal room-temperature magnetic moment[6] ($\mu_{eff} = 1.78$ BM).

$$[(phen)_2Fe(Cl)-O-Fe(Cl)(phen)_2]Cl_2$$

The nmr isotropic shifts of these complexes were measured in D_2O at 28° at 60 MHz and are reported in Table I. Of interest is the fact that distinct signals were found for all four protons. Here, save for the 2,9-proton resonance, the half-widths are between 200 and 400 Hz.[9] In low-spin $Fe(phen)_3(ClO_4)_3$ and related complexes[10] the observed half-widths, 40–80 Hz, are much smaller as would be expected and provide further evidence that the binuclear complexes discussed herein do not contain low-spin iron(III). High-spin octahedral d^5 complexes are expected to be magnetically isotropic, and thus we can disregard the pseudocontact term and can attribute the isotropic shifts to a contact interaction only. If J is approximately 100 cm^{-1}, about 50% of the molecules at any time will be in the diamagnetic, $S' = 0$ ground state. Most of the remaining molecules will have energy $2J$, spin $S' = 1$,[11] and will be responsible for the contact shifts.

Table I. Nmr Isotropic Shifts[a]

Compound	$\Delta\nu_{2,9}$	$\Delta\nu_{3,8}$	$\Delta\nu_{4,7}$	$\Delta\nu_{5,6}$
$[Fe_2(phen)_4O]Cl_4 \cdot 5H_2O$	−1320	−525	+21	−180
$[Fe_2(4,7\text{-dmp})_4O]Cl_4 \cdot 3H_2O$		−552	(−9)	−210
$[Fe_2(5,6\text{-dmp})_4O]Cl_4 \cdot 4H_2O$		−560	+20	(−22)

[a] The shifts are in cps relative to the appropriate diamagnetic tris-iron (II) complex. Methyl resonances are enclosed in parentheses.

As will be noted, the contact shifts for $[Fe_2(phen)_4O]Cl_4$ follow the pattern (attenuation) expected for a σ mechanism.[12] The very small positive value for $\Delta\nu_{4,7}$ could be explained by a small contribution from a spin polarization mechanism or an extremely minute contribution from a π-delocalization mechanism, which for all practical purposes would be nil. The contact shifts of the methyl-substituted complexes provide further substantiation for a σ-delocalization mechanism because of the small negative values of the methyl proton contact shifts. We have discounted an alternative mechanism whereby the contact shifts in these complexes are due to a combination of both σ and π delocalization such that at the 4,7 protons these two effects numerically cancel each other, causing $\Delta\nu_{4,7} \cong 0$ for $[Fe_2(phen)_4O]Cl_4$. Were this true, one would expect that in $[Fe_2(4,7\text{-dmp})_4O]Cl_4$, the 4,7-$CH_3$ shift would be at least −200 cps owing to the domination of the π mechanism at the 4,7-methyl position. It seems likely that the unpaired spin resides in an orbital of σ symmetry localized mainly on the Fe–O–Fe unit and is then *via* σ bonding partially transferred into the highest filled phenanthroline σ molecular orbital. The success of observing and interpreting the nmr spectra of these complexes means that similar studies can be extended to binuclear oxo-bridged iron(III) species of biochemical significance such as μ-oxo-bis(tetraphenylporphyrin)iron(III).[13]

Such work is at present being initiated in this laboratory along with a study of other binuclear iron(III) complexes in order to ascertain if a σ-delocalization mechanism is prevalent in these complexes.

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The Thiophilic Addition of Phenyllithium to Thiobenzophenone

Sir:

Stabilization of an anion or radical by a sulfur atom adjacent to the site of formal electron localization is well documented.[1] Nucleophilic attack on sulfur has also been studied extensively in recent years.[2] We

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