mp 134–136°, which was hydrolyzed to the all-*cis*-diketone IV,[4] mp 78–80°. With acetic anhydride and sodium acetate, IV afforded the diacetate Va,[4] mp 114–115°, while with NaH, followed by acetic acid, IV was converted to the hydroquinone Vb, mp 163–166°. Compound Vb could be oxidized with N_2O_4 to the corresponding quinone, 2,3-(*cis*-3,4-dimethylcyclobuteno)-1,4-naphthoquinone,[4] mp 99–100°.

With NaH in tetrahydrofuran, IIIa was converted to the dienolate, but on quenching in H_2O this produced a hydroquinone Ib which rapidly formed a dimer.[4] Accordingly, the photoadduct IIIb[4,5] from naphthoquinone and diphenylacetylene was prepared, and converted to the dark green dienolate ion with NaH in tetrahydrofuran (or KO-*t*-Bu in dimethyl sulfoxide). With acetic anhydride this solution afforded a 95% yield of the diacetate[4] of Ia, mp 237–238°, while with methyl iodide it yielded chiefly the C-methylation product IIIc.[4,5] On quenching with aqueous NH_4Cl the dianion afforded the hydroquinone Ia,[4] which slowly went to three dimers,[4] mp 217–218°, mp 239–241°, and mp 229–231°, in 78% overall yield, together with *ca.* 20% recovered IIIb.

The hydroquinone Ia (1 mg) was quickly dissolved in 10 ml of ethanol and 10 ml of aqueous 0.2 *N* acetate buffer, pH 5.6. Dc polarography with a dropping mercury electrode shows a two-electron oxidation wave at $E_{1/2}$ 0.163 V (sce). By contrast, under the same conditions naphthohydroquinone has $E_{1/2}$ −0.113 V, while Vb has $E_{1/2}$ −0.153 V. The diffusion current for Ia decreased *ca.* 20% in 30 min presumably because of dimerization; however, initially the diffusion current corresponded to 75% of that expected for Ia, and the dimers and diketone IIIb were shown not to be responsible for the electrochemistry at 0.163 V.

The product IIa is even more unstable than Ia. IIa can be generated independently by treatment of IIId,[4] mp 173–175°, the photoadduct of diphenylacetylene and 2-chloronaphthoquinone, with NaH in tetrahydrofuran, but it yields only a dimer,[4] or a 1:1 adduct,[4] mp 289–291°, if the elimination is performed in the presence of diphenylisobenzofuran. The rapid dimerization of IIa makes the oxidation of Ia slightly irreversible electrochemically.

In order to confirm our assignment of potentials, we have also prepared the dianion of Ia from IIIb and NaH as described above, and examined its electrochemistry in purified anhydrous dimethylformamide with 0.1 *M* n-$Bu_4N^+ClO_4^-$ as electrolyte. A three-electrode-controlled potential instrument was used, with a platinum spiral anode, a dropping mercury cathode, and a calomel reference electrode isolated by a fine-porosity sintered disk. Under these conditions the dianion of Ia showed two one-electron oxidation waves, at −0.86 and −0.40 V (max). Naphthohydroquinone dianion had oxidation waves at −1.32 and −0.63 V (max), and naphthoquinone showed reduction waves at −0.65 and −1.42 V. The diffusion current due to the dianion of Ia diminished with time by irreversible destruction of the compound.

Thus both Ia and its dianion are more difficult to oxidize than is naphthohydroquinone or its dianion. By contrast, the cyclobutene analog Vb is more easily oxidized than naphthohydroquinone, since the slight strain effect[6] does not quite cancel the small substituent effect on the oxidation potential. The 0.27-V difference in oxidation potential between Ia and naphthohydroquinone corresponds to 12.4 kcal/mol, while the 0.31-V difference between I and Vb corresponds to 14.2 kcal/mol. The data on the dianions correspond to a 16 kcal/mol difference in the $\Delta G°$ for oxidation of Ia and of naphthohydroquinone dianions.

Obviously the irreversible dimerization of IIa affects these data, although the direction is such as to underestimate the antiaromaticity effect in IIa. Furthermore, small strain[6] and substituent effects must also be playing some role in the comparison of Ia with Vb. However, it seems clear that the bulk of the increased difficulty in oxidizing Ia is due to the antiaromaticity of the cyclobutadiene ring in IIa. Such antiaromaticity should raise the energy of Ia as well as IIa, although to a lesser extent, so our measurement reflects only a portion of the destabilization in a cyclobutadiene ring (as did our previous case,[3d] in which a 15 kcal/mol antiaromatic effect was seen from partial cyclobutadiene character). Thus the full antiaromatic destabilization of cyclobutadiene probably involves considerably more than the 12–16 kcal/mol of our present measurement.

(6) *Cf.* the small Mills–Nixon effect in the quinone redox potentials studied by R. T. Arnold and H. E. Zaugg, *J. Amer. Chem. Soc.*, **63**, 1317 (1941).

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Received April 4, 1970

Photooxidation of α-Chymotrypsin Sensitized by the Inhibitor N-Acetyl-3-nitrotyrosine

Sir:

Dye-sensitized photooxidation of proteins has been shown to be a valuable tool either for probing the degree of exposure of the photooxidizable amino acid residues[1,2] or for mapping the location of specific residues in predetermined regions of protein molecules.[3–5] The latter procedure involves the irradiation of proteins containing a linked sensitizer, so that only the amino acid side chains adjacent to the sensitizer can be modified. In this paper we describe a modification of this procedure which should enlarge the scope of the method and specifically direct the photooxidative attack toward the active site of enzymes.

This goal is achieved by using a reversible inhibitor of the given enzyme as the sensitizer. Although the complexed inhibitor is in equilibrium with unbound molecules, the concentration of the sensitizer in the catalytic region is comparatively very high; consequently, the photooxidation rate of the susceptible residues located in this region should be by far greater than that of the other susceptible residues which have a smaller probability of interacting with the free in-

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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.

Acknowledgment. This work was financially supported by the Consiglio Nazionale delle Ricerche.

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Received February 27, 1970

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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