THE MECHANISM OF REACTIVATION OF PHENYLMETHANESULFONYL α-CHYMOTRYPSIN

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Studies of the reaction of sulfonyl fluorides with enzymes of the "serine esterase" class have shown that these substances may be potent irreversible inhibitors (1). Further work on the reaction of phenylmethanesulfonyl fluoride (PMSF) with α-chymotrypsin indicates that a selective sulfonylation of the enzyme occurs (2). A single sulfonyl group is introduced into the enzyme molecule, even at high concentrations of inhibitor, resulting in total loss of enzymic activity; chymotrypsinogen and DFP inhibited chymotrypsin fail completely to react with PMSF-C^{14}. The liberation of exactly one equivalent of hydrogen ion when the reaction is carried out at pH 7.4 strongly suggests that the site of sulfonylation is the hydroxyl group of the active-site serine residue. These results are analogous to the well-known reaction of chymotrypsin with diisopropyl phosphorofluoridate (DFP).

When phenylmethanesulfonyl-7-C^{14} chymotrypsin is incubated at 40° in the presence of various buffers, the sulfonyl group is indefinitely stable between pH 4 and 8.5; however, it is rapidly lost at lower or higher pH values. At pH 2.0 in 0.04 M NaCl protein-bound radioactivity is lost in a first-order reaction having a rate constant of 2.9×10^{-2}

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min.$^{-1}$. At 25° under similar conditions no detectable reaction occurs. In marked contrast to the reaction in aqueous solution desulfonylation is a rapid process in 8 M urea at 10°. The rate constant is independent of pH over the range 2 to 8.5 but increases at higher pH. These results indicate that desulfonylation is hindered in the compactly folded protein (3,4).

PMS-chymotrypsin may be reactivated to a large extent. When the sulfonyl enzyme is desulfonylated in dilute solution (pH 2.0, 40°, 0.04 M NaCl) the resulting protein is enzymically inactive. However, if this product is allowed to stand at pH 7 it regains ca. 60% of the enzymic activity* of native chymotrypsin within four hours. Recovery of potential activity at pH 2.0 is roughly first-order with a rate constant of 1.7×10^{-2} min.$^{-1}$. Under the conditions of this experiment native chymotrypsin is stable and shows full activity immediately. If desulfonylation is conducted in concentrated protein solution reactivation cannot be observed due to irreversible denaturation.

The above data are consistent with the hypothesis set forth in Figure 1. The facile reaction of N-acyl-O-sulfonylethanolamines to yield 2-oxazolines is well-known (5). Since this reaction has rigid steric requirements it is not surprising that it occurs only in partially unfolded forms of PMS-chymotrypsin. Equally familiar is the opening of the oxazoline ring in acid solution to yield an O-acylethanolamine (6). This ester is stable in dilute acid but in neutral solution undergoes the O,N-acyl shift, resulting in formation of an N-acylethanolamine. The ester III corresponds to the enzymically inactive protein produced by desulfonylating at pH 2, while the end product IV is apparently native chymotrypsin.

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* Enzymic activity was assayed spectrophotometrically using N-acyl-L-tyrosine ethyl ester as substrate. See ref. 1.

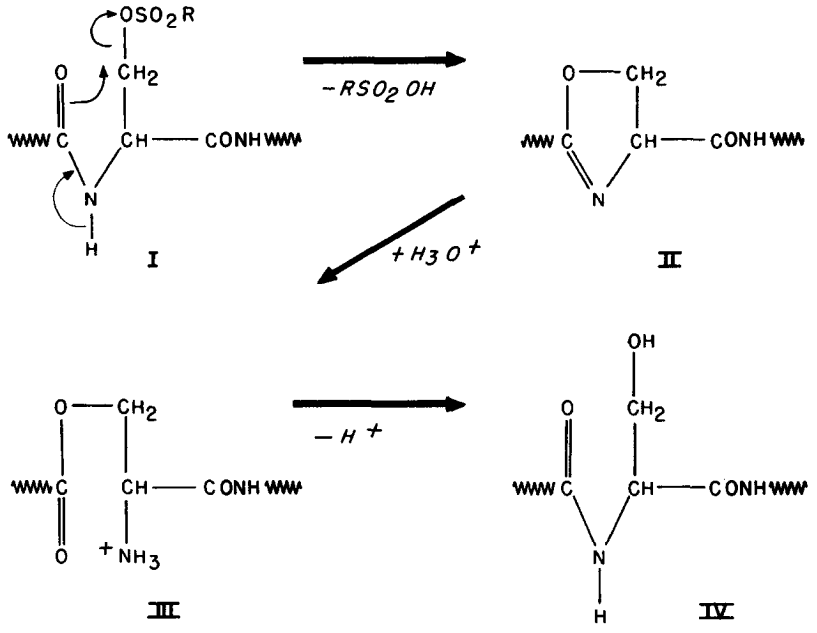

Figure 1

The hypothesis was tested by treating the "ester" protein III with fluorodinitrobenzene (FDNB), hydrolyzing the derivative, and analyzing the DNP-amino acids by means of two-dimensional paper chromatography (7). PMS-chymotrypsin was chromatographed on CM-cellulose to remove N-terminal serine impurities. Desulfonylation was carried out in a pH-stat with 12 mg. of PMS-chymotrypsin in 3 ml. of 0.04 M NaCl, pH 2.0, at 40° for 3.5 hours. Loss of sulfonate is complete under these conditions. Guanidine hydrochloride (4.1 g., final concentration 6 M), 1 ml. of ethanol, and 0.1 ml. of FDNB were then added. The pH was adjusted to 7.0 with M KOH and the reaction allowed to proceed at this pH for 40 minutes at 40°. Subsequent isolation and hydrolysis were performed essentially as described by Smillie and Neurath (8). The yield of DNP-serine was satisfactory (Table 1)*. Further support for the hypothesis comes from a modification

* Variable amounts of DNP-serine have been isolated in experiments using lyophilized samples of "ester" protein which were prepared in dilute solution and which can be reactivated at pH 7.

of the experiment in which the desulfonylated protein was allowed to stand in guanidine solution at pH 7.0 for 4 hours, 25°, prior to adding ethanol and FDNB. In this case only a trace of DNP-serine was detected. Control experiments with chromatographed diisopropylphosphoryl chymotrypsin show that N,O-acyl shifts apparently do not occur under the conditions of desulfonylation.

TABLE I

Yields of DNP-amino acids from chymotrypsin derivatives†

Protein	Treatment	% DNP Ala	% DNP Ileu	% DNP Ser
PMS-chtr	pH 2	54*	46*	22*
PMS-chtr	(a) pH 2; (b) pH 7	56	44	1.5
DIP-chtr	none	54	46	0
DIP-chtr	pH 2	56	42	0

† Yields are uncorrected. Extinction coefficients are taken from ref. 7. Ether-extractable DNP-amino acids from the hydrolysate of 10 mg. of DNP-protein were chromatographed.

* Average of three experiments; other values are average of duplicate experiments. Average deviations do not exceed 10% of stated values.

The demonstration of a free amino group of serine in the desulfonylated protein lends support to the hypothesis presented here. The intermediate oxazoline II is identical to that proposed by Rydon (9) as the active species of chymotrypsin. Since it is unstable under conditions where active chymotrypsin is stable, it may be excluded as a possibility. The ester III is a unique material and may prove useful for splitting the aspartyl serine peptide bond in the active site of chymotrypsin.

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