The reaction of DFP* with trypsin

The reaction of inhibitors of the type $X-\underset{\underset{O}{\|}}{P}-(OR)_2$ with cholinesterase and the proteolytic enzymes chymotrypsin and trypsin has been intensively studied with a view to determining the nature of the group with which the inhibitor combines and which is essential for enzymic activity. It has been found[1,2] that certain nucleophilic reagents (nicotinohydroxamic, picolinohydroxamic acid and their methiodides) can reverse this inhibition by displacing the $(RO)_2-\underset{\underset{O}{\|}}{P}-$group from the protein. However, the observation that reactivation is dependent upon the age of the preparation[1,2] has led to the suggestion that the inhibition is a two-phase process involving an initial phosphorylation of one group in the protein to form a complex labile to reactivator, followed by a shift of the alkyl phosphoryl group to a second linkage, stable to reactivator, according to the following scheme:

$$E + DFP \rightleftharpoons E \cdot DFP \longrightarrow \underset{\text{(unstable)}}{E_1DIP} + HF$$
$$\downarrow$$
$$\underset{\text{(stable)}}{E_2DIP}$$

Degradation studies upon the inactive DIP-derivatives of chymotrypsin, trypsin and cholinesterase have shown in each case that the hydroxyl group of serine is phosphorylated[3,4,5]. In general the age of the preparation (*i.e.*, after inhibition) was such that the shift must be assumed to have been complete at the outset of degradation. The evidence is, therefore, strong that serine is the residue involved in stable linkage with DIP (*i.e.*, E_2DIP in the above scheme).

Various lines of evidence have indicated an involvement of the imidazole side chain of histidine in the catalytic activity of these enzymes and the implication of imidazole-DIP as the E_1DIP complex above is consistent with the following data:

(1) The pH dependence of DFP inhibition[6] indicates the involvement of an ionizing group with a pK of 5–7.

(2) A study of the reaction of DFP with free amino acids[7] has shown that while no stable phosphorylated derivatives were produced, histidine and tyrosine were able to catalyze the hydrolysis of DFP, indicating the formation of unstable DIP-amino acid complexes as intermediates; serine was inactive under these conditions. DIP-imidazole was synthesized under anhydrous conditions and found to be extremely unstable in aqueous solution (half-time at 30° C and pH 7.4 = 2.7 hours).

(3) Histidine derivatives have been found to accelerate the hydrolysis of *p*-nitrophenyl acetate[8], an inhibitor similar to DFP which inhibits chymotrypsin by the acetylation of some group in the active center.

Despite the postulated shift of the di*iso*propyl phosphoryl group, the enzyme remains inactive so that serine is either part of the active center or so close to it that the large DIP group can maintain inhibition by steric hindrance. The maintenance of the serine group in this favorable position for phosphorylation could be the result of:

(a) The two residues being close to each other in the polypeptide sequence;

(b) The residues being brought into juxtaposition by their presence in adjacent turns of an α-helix or by the close apposition of two separate parts of an α-helix (or two separate α-helices).

The study of serine peptides resulting from the degradation of DIP-enzymes (chymotrypsin and trypsin) has not so far revealed histidine as a constituent so that (a) is unlikely. If (b) were true then it might be expected that if the protein were unfolded in strong urea immediately following reaction with DFP, the shift of the DIP group might be interfered with by removal of serine from the immediate neighborhood of the imidazole-DIP complex. In this case it would be expected that by analogy with synthetic DIP-imidazole, which is extremely unstable, the DIP-imidazole on the protein would decompose to free DIP, leaving the protein-bound imidazole free and the serine unphosphorylated.

In order to facilitate the detection of unstable DIP-enzyme derivatives a new technique has been developed whereby the phosphorylated enzyme can be rapidly separated from excess DFP and its hydrolysis product DIP. Use is made of a column of Dowex-50 × 2 cation exchange resin, 200–400 mesh, in the NH_4^+ form. The column (12 × 0.9 cm) is packed in a polyethylene tube and pre-treated with 0.1 M sodium citrate buffer pH 3.0; an aliquot of the reaction mixture of the enzyme with $DF^{32}P$ is then applied to the top of the column and forced through under

* The following abbreviations will be used herein: DFP = di*iso*propylphosphofluoridate; DIP = di*iso*propyl phosphoryl; BAEE = benzoyl-arginyl-ethyl ester.

pressure. Upon elution with 0.1 *M* acetic acid, DIP and DFP are rapidly eluted, being clearly separated from each other, while the labelled protein is retained at the top of the resin and may be located by monitoring the column and then cutting through column and resin in that region and eluting the labelled protein by suspension of the resin in 1 *N* sodium hydroxide. Dialysis experiments were also carried out and the results obtained by both techniques are summarized in Table I.

TABLE I

THE LABELLING OF TRYPSIN BY $DF^{32}P$

Experimental conditions	*Per cent theoretical labelling with ^{32}P*
1. Control reaction of trypsin*, followed by	
(a) Dialysis against 0.001 *N* HCl	100 (35,000 c.p.m./mg trypsin)
(b)** Dialysis against 8 *M* urea in 0.004 *M* sodium phosphate, pH 7.6	95.8
2. Reaction of trypsin as above but initially allowed to unfold in 8 *M* urea, pH 3.0 followed by reaction at pH 7.6 in 8 *M* urea.	
(a) Dialysis against 0.001 *N* HCl	3.6
(b) Dialysis against 8 *M* urea in 0.004 *M* sodium phosphate, pH 7.6	1.0
(c) Protein separated on Dowex-50 as described in the text after the following periods of reaction with $DF^{32}P$	
68 min	0.97
1547 min	1.15
3. Reaction with DIP-trypsin, 3 × recrystallized (separated on Dowex-50)	0.36
4. Reaction with trypsinogen (dialyzed against 0.001 *N* HCl)	1.5

* Reaction conditions were 10-fold molar excess of $DF^{32}P$, 0° C, pH 7.6 for 60′ unless otherwise stated. Dialysis was for 36 hours at 30° C with several changes. Trypsin (2 × crystallized) and trypsinogen (1 × crystallized) were obtained from Worthington Biochemical Corp. and dialyzed exhaustively against 0.001 *N* HCl to remove salt.

** The protein solution was made 8 *M* in urea before starting the dialysis.

It is clear that exhaustive dialysis of freshly inhibited DIP-trypsin in the presence of 8 *M* urea at 30° C does not liberate a significant amount of DIP; esterase measurements confirm this in showing no restoration of enzymic activity. This would negate the possibility that a significant portion of the DIP is bound in a form as labile as DIP-imidazole after 60 minutes reaction. It might be mentioned that this period is short in comparison with the time during which chymotrypsin (1) and cholinesterase (2) can be reactivated.

When, however, the protein is initially unfolded in 8 *M* urea and then reacted (still in 8 *M* urea) the dialysis experiments indicate a negligible degree of labelling. Similarly, activity measurements made during the course of the reaction by one hundred fold dilution of aliquots with 0.001 *N* HCl show that no progressive inhibition of enzymic activity occurs, thus indicating that the usual course of labelling and inhibition by DFP is interrupted by 8 *M* urea.

This lack of reactivity of $DF^{32}P$ in the presence of 8 *M* urea, coupled with the lack of esterase activity under these conditions* lends support to the idea of HARTLEY AND KILBY[8] that DFP is,

* When trypsin is unfolded in 8 *M* urea, no esterase activity can be detected as long as the protein remains in 8 *M* urea (*i.e.*, the substrate BAEE is also made in 8 *M* urea), but as soon as the urea solution of the protein is extensively diluted then activity is fully restored[9]. VISWANATHA AND LIENER have stated that trypsin remains active in 8 *M* urea[10] but only on the basis of undiminished activity after dilution.

These authors have also recently reported[11] that diethyl-*p*-nitrophenyl-phosphonate reacts in urea with trypsin at a site which can be distinguished from the site responsible for proteolytic activity, and that diethyl phosphoryl trypsin could be partially reactivated by treatment with urea. No explanation will be offered at this time for the discrepancy between these results and those of the present study, in which the direct uptake of phosphate rather than the liberation of the unbound reaction product (*p*-nitrophenol) was used as a criterion for phosphorylation.

in fact, a special substrate and can only react under conditions when the enzyme is active. In support of this conclusion, trypsinogen was found not to be labelled by $DF^{32}P$.

The rapidity of separation of labelled trypsin and free DFP (and DIP) on the Dowex-50 column (5–7 minutes) enabled a search to be made for an E_1DIP complex as postulated above, but none could be detected either during the reaction with trypsin in the presence of 8 *M* urea nor when "cold" DIP-trypsin (3 times crystallized) was treated with $DF^{32}P$. In the first case, both sites E_1 and E_2 would be available, since no substitution had occurred; while in the case of DIP-trypsin, since any shift must have been completed during the lengthy preparation and crystallization of the protein, the E_1 site would be unoccupied and therefore available for further phosphorylation. Under conditions, therefore, where the imidazole side chains of trypsin are available but where the usual course of inhibition finally resulting in DIP stably bound to serine is prevented, no labelling of the protein is observed even though the analytical technique would ensure the detection of very labile intermediates. Moreover, even if the intermediate existed only on the surface of the enzyme and were rapidly decomposed by water to form free DIP and enzyme, a DFP-ase effect of trypsin would be manifest by an increased formation of DIP. Comparison with a control in which the same amount of DFP was incubated in 8*M* urea under the same conditions of temperature and pH showed, however, no difference in the amount of DIP produced.

In summary, therefore, it may be said that no evidence has been found to support the hypothesis that the phosphorylation of imidazole constitutes the initial stage of the combination of DFP with trypsin.

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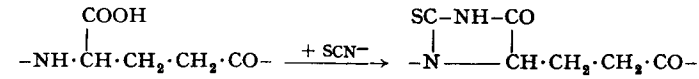
Condensation of γ-glutamyl peptides with thiocyanate*

Ammonium thiocyanate in the presence of acetic anhydride has been used as a reagent for the determination of C-terminal amino acids in proteins or peptides[1–5]. Using this reaction we found that proteins combine with 10–20 $S^{14}CN$-residues per 10^5 g of protein[6–8]. The radioactive $S^{14}CN$-residues were not exchanged with nonradioactive thiocyanate; this suggested binding by covalence, and seemed to indicate the combination of SCN-residues with the free α-carboxyl groups of γ-glutamyl or β-aspartyl residues in the peptide chain; this reaction would result in the formation of thiohydantoin rings in the chain:



Our results did not exclude, however, the possibility that α-glutamyl residues are converted into γ-glutamyl residues under the drastic conditions of the treatment with thiocyanate. Rearrangements of this type have been described[9–11]. We examined, therefore, the reaction of α- and γ-polyglutamic acid with thiocyanate.

Radioactive ammonium thiocyanate was prepared from $Na^{14}CN$ and sulfur and recrystallized from ethanol with added nonradioactive ammonium thiocyanate as carrier. Polyglutamic acid preparations were mixed with the radioactive thiocyanate, acetic anhydride and acetic acid as