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# Inactivation of interferons: Halomethyl ketone derivatives of phenylalanine as affinity labels

[hydrophobic site/protease inhibitor/essential histidine(s)/active site]

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ABSTRACT **Antiviral activity of rabbit and mouse fibroblast interferons was irreversibly destroyed by treatment with halomethyl ketone derivatives of phenylalanine but not by treatment with a halomethyl ketone derivative of lysine. The inactivation reaction was pH dependent, suggesting the involvement of an amino acid residue ionizing in the region of pH 7. Tryptophan and phenylalanine, known ligands of interferons, protected rabbit interferon substantially against inactivation by the chloromethyl ketone derivative of *N*-tosylphenylalanine. Mixed bovine brain gangliosides protected rabbit and mouse interferons against inactivation by this reagent. Although halomethyl ketone derivatives of phenylalanine were originally designed and used for affinity labeling of the active site of chymotrypsin and similar enzymes, no evidence was found for a chymotrypsin-like activity of interferons. It is proposed that halomethyl ketone derivatives of phenylalanine inactivate interferon by an affinity labeling mechanism, first binding to a hydrophobic binding site and then reacting irreversibly with a nearby nucleophilic amino acid residue, which appears to be a histidine. This conclusion implies that a hydrophobic site on interferons is necessary for their antiviral activity.**

Interferons are proteins secreted by animal cells in response to virus infection or other stimuli. They are capable of binding to cells and inducing a state in which the cells do not support virus replication.

Interferons are also known to bind to various ligands, including aromatic amino acids, anionic polyaromatic dyes, polyribonucleotides, and gangliosides (1). It has been suggested that the binding of gangliosides takes place at a site on the interferon molecule involved in binding to the cell surface (2). The biological significance of the other ligand affinities of interferons is not clear.

We have recently presented evidence that the binding of fibroblast interferons to hydrophobic ligands, especially aromatic substances, may reflect a hydrophobic binding site essential for antiviral activity. This suggestion was based on our finding that tryptophan, a known ligand of interferons (3), protects mouse and rabbit fibroblast interferons (MuIFN and RaIFN, respectively) against the arginine-specific reagent butanedione (4). As an extension of this work, we have now applied the technique of affinity labeling (5) using molecules containing a moiety able to bind hydrophobically to interferons and a moiety able to react with nearby nucleophilic amino acid side chains. In this paper, we report the specific inactivation of RaIFN and MuIFN by halomethyl ketone derivatives of phenylalanine. This inactivation has been characterized, and evidence is presented that the reagents attach to a hydrophobic binding site by an affinity labeling mechanism and that the inactivation is due to a specific modification of histidine residues. Since these phenylalanine derivatives were originally designed to be active-site reagents for chymotrypsin and related enzymes (6), attempts were made to test the possibility that interferons are proteases similar to chymotrypsin.

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## MATERIALS AND METHODS

**Chemicals.** $N^{\alpha}$-Tosyl-L-phenylalanine chloromethyl ketone (Tos-Lys$CH_2Cl$), $N^{\alpha}$-tosyl-L-lysine chloromethyl ketone (Tos-Phe$CH_2Cl$), $N^{\alpha}$-carbobenzoxy-L-phenylalanine chloromethyl ketone (Cbz-Phe$CH_2Cl$), diisopropyl fluorophosphate, and cytochrome *c* were purchased from Serva Biochemicals (Heidelberg, Federal Republic of Germany). $N^{\alpha}$-Carbobenzoxy-L-phenylalanine bromomethyl ketone (Cbz-Phe$CH_2Br$) was obtained from Nutritional Biochemicals. Phenylmethylsulfonyl fluoride and mixed bovine brain gangliosides, type III, were from Sigma. L-Tryptophan, L-phenylalanine, and bromoacetic acid were Merck products. Iodoacetic acid from Fluka was recrystallized three times before use. Poly(I) and poly(I-C) were Boehringer Mannheim products.

**Interferons.** Rabbit interferon was prepared by inducing primary rabbit kidney cultures with poly(I-C) using a superinduction schedule as described (7). The interferon-containing culture fluids were concentrated to 0.03–0.017 vol by pressure ultrafiltration with an Amicon PM-10 membrane. Concentrated RaIFN was dialyzed exhaustively against either 0.02 M barbital buffer, pH 7.5/0.15 M NaCl or a 1:5 dilution of phosphate-buffered saline (pH 7.2) in water. This crude concentrated dialyzed RaIFN was incubated at 37°C overnight before use in inactivation experiments. Control experiments showed no significant effects of such incubation on RaIFN antiviral activity. Such preparations of RaIFN contained protein at 1–2 mg/ml and had titers in our assay of $1–2 \times 10^4$ units/ml.

MuIFN was a partially purified preparation obtained from Calbiochem-Behring. It had a protein content of 0.041 mg/ml and an interferon titer of $1.0 \times 10^6$ units/ml.

**Assay of Interferons.** A semimicro cytopathic effect test with 1:2 dilution steps was used to assay all interferons (1). For RaIFN, we used rabbit kidney RK 13 cells and vesicular stomatitis virus; for MuIFN, L cells and encephalomyocarditis virus were used. In all assays, the samples (0.02 ml) were diluted 1:50 before starting the 1:2 dilution series. For each sample, four rows of wells in a microtiter plate were used. Each point or number in the results represents the mean of duplicate samples.

Abbreviations: Tos-Phe$CH_2Cl$, $N^{\alpha}$-tosyl-L-phenylalanine chloromethyl ketone; Tos-Lys$CH_2Cl$, $N^{\alpha}$-tosyl-L-lysine chloromethyl ketone; Cbz-Phe$CH_2Cl$, $N^{\alpha}$-carbobenzoxy-L-phenylalanine chloromethyl ketone; Cbz-Phe$CH_2Br$, $N^{\alpha}$-carbobenzoxy-L-phenylalanine bromomethyl ketone; RaIFN, rabbit fibroblast interferon; MuIFN, mouse fibroblast interferon.

**Inactivation Procedures.** All inactivation reactions were done in 1.5-ml Eppendorf reaction tubes with total volumes of 0.05 ml in most experiments and 0.1 ml in the others. The alkylating reagents were dissolved in methanol just before use. Generally, the reaction mixtures were constituted as follows: 1 vol of interferon, 1 vol of buffer, 0.25 or 0.5 vol of alkylating reagent and, when appropriate, 0.25 vol of water or methanol. The buffer used was normally 0.5 M sodium phosphate (pH 8.0). In some experiments, 0.1 M sodium phosphate (pH 8.0) or 0.02 M barbital, pH 8.0/0.15 M NaCl was used. The final concentration of methanol in the incubation mixtures was 10% or 20%. After mixing, the reaction mixtures were incubated at 37°C. Although we initially stopped the reactions by adding excess dithioerythritol, we found that simple cooling in an ice-water bath and immediate assay were just as effective. In all experiments reported here the values given are relative to controls, which were treated exactly like the experimental samples except for the lack of inactivation reagent.

In all inactivation experiments with RaIFN, the crude concentrated dialyzed material was diluted 1:1 or 1:4 with water before use. All incubation mixtures for inactivation of MuIFN contained cytochrome *c* at 1 mg/ml as a stabilizing agent.

In protection experiments, reaction mixtures containing all components except for chloromethyl ketone were first incubated at 37°C for 10 min and then treated with Tos-PheCH$_2$Cl, mixed, and incubated as described above.

## RESULTS

**Inactivation of Interferons by Halomethyl Ketone Derivatives of Phenylalanine.** Initial experiments showed that Tos-PheCH$_2$Cl, but not Tos-LysCH$_2$Cl, progressively inactivated crude RaIFN. This inactivation was slow, requiring ≈20 hr of incubation at 37°C with 1 mM Tos-PheCH$_2$Cl for 80–90% inactivation. However, it could be shown that the incubation time necessary for nearly complete inactivation was drastically decreased if, before Tos-PheCH$_2$Cl treatment, the diluted RaIFN was incubated at 37°C overnight. Control experiments indicated that no loss of antiviral activity occurred on incubation without Tos-PheCH$_2$Cl. The nature of this activation phenomenon is not clear. MuIFN did not require prior incubation to obtain rapid inactivation by halomethyl ketone derivatives of phenylalanine.

With previously incubated RaIFN, inactivation by 1 mM Tos-PheCH$_2$Cl was rapid; 80–90% inactivation was achieved within 2 hr at 37°C (Fig. 1). In contrast to the effect of Tos-PheCH$_2$Cl, there was no significant inactivation of RaIFN by Tos-LysCH$_2$Cl during a 2-hr incubation at 37°C. This type of experiment has been repeated several times with similar results.

Inactivation of RaIFN by Tos-PheCH$_2$Cl was concentration dependent (Fig. 2). Included in this figure are data from a similar experiment showing that identical concentrations of Tos-LysCH$_2$Cl did not affect the antiviral activity of RaIFN under these conditions. The imprecision of the interferon assay did not allow the type of kinetic analysis essential to demonstrate a saturation effect (5).

To test the specificity of the alkylating reagents, we have compared the effects of Tos-LysCH$_2$Cl and Tos-PheCH$_2$Cl with those of the Tos-PheCH$_2$Cl-related compounds Cbz-PheCH$_2$Cl and Cbz-PheCH$_2$Br, as well as two more commonly used alkylating agents, bromoacetic acid and iodoacetic acid. Cbz-PheCH$_2$Cl, which is identical to Tos-PheCH$_2$Cl except for the amino blocking group, and its bromo analogue Cbz-PheCH$_2$Br inactivated RaIFN in a similar manner. In contrast, bromoacetic acid and iodoacetic acid did not inactivate RaIFN under conditions that gave rapid inactivation by Tos-PheCH$_2$Cl (Table 1).

Partially purified MuIFN was also inactivated specifically by

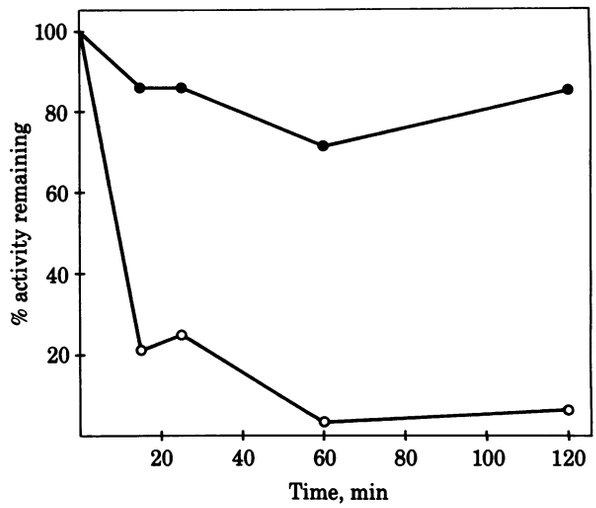


FIG. 1. Effects of 1 mM Tos-LysCH$_2$Cl (●) and Tos-PheCH$_2$Cl (○) on antiviral activity of RaIFN. RaIFN was in barbital buffer and 0.5 M sodium phosphate (pH 8.0) was used in the reaction mixtures. The final concentration of methanol was 20%.

Tos-PheCH$_2$Cl with no significant inactivation by Tos-LysCH$_2$Cl (Table 1). As mentioned above, prior incubation was not necessary for rapid inactivation. Cbz-PheCH$_2$Cl and Cbz-PheCH$_2$Br also inactivated MuIFN. Cbz-PheCH$_2$Cl was less effective than Tos-PheCH$_2$Cl, whereas Cbz-PheCH$_2$Br appeared to be as effective as Tos-PheCH$_2$Cl, consistent with the expected greater reactivity of the bromomethyl ketone group compared with the chloromethyl ketone group. Bromoacetic acid and iodoacetic acid also failed to inactivate MuIFN under these conditions (Table 1).

**pH Dependence of Inactivation.** In an attempt to discover the type of amino acid residue modified by the halomethyl ketones of phenylalanine, we studied the pH dependence of the inactivation. Tos-PheCH$_2$Cl inactivation of RaIFN was highly dependent on pH (Fig. 3). Inactivation was maximal above pH 8 and minimal at pH 5. The pH values for 50% inactivation ranged from 6.65 to 7.15 in a series of three experiments, with

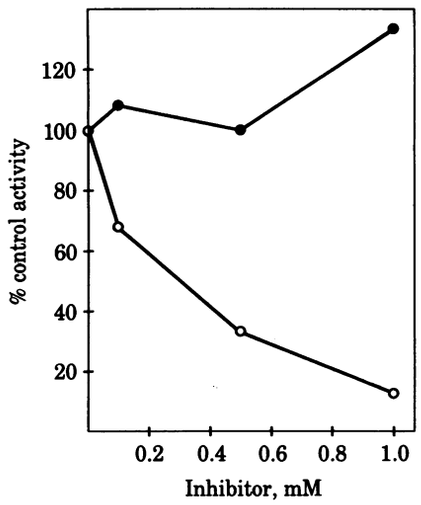


FIG. 2. Concentration dependence of the inactivation of RaIFN by Tos-PheCH$_2$Cl (○) and the lack of effect of Tos-LysCH$_2$Cl (●). RaIFN was in barbital buffer. Reaction mixtures contained 0.02 ml of RaIFN solution, 0.06 ml of barbital buffer (0.02 M, pH 8.0/0.15 M NaCl), and 0.02 ml of methanol containing various concentrations of Tos-LysCH$_2$Cl or Tos-PheCH$_2$Cl. Incubation was for 6 hr at 37°C.

Table 1. Specificity of interferon inactivation by alkylating reagents

| Addition | % activity RaIFN* | % activity MuIFN† |
|---|---|---|
| None | 100 | 100 |
| Tos-Lys$CH_2Cl$ | 100 | 111 |
| Tos-Phe$CH_2Cl$ | 14 | <5 |
| Cbz-Phe$CH_2Cl$ | 28 | 28 |
| Cbz-Phe$CH_2Br$ | 33 | <5 |
| Bromoacetic acid | 78 | 100 |
| Iodoacetic acid | 111 | 89 |

Additions were made at 1 mM. The buffer was 0.1 M sodium phosphate (pH 8.0), and all reaction mixtures contained 20% methanol.
* RaIFN in phosphate-buffered saline/water (1:5); incubation, 60 min; 37°C.
† Incubation, 20 min; 37°C.

a mean of 6.97. Qualitatively similar results were obtained for inactivation of MuIFN by Tos-Phe$CH_2Cl$ (data not shown). Although this approach is indirect and suffers from the ambiguities involved in studying the ionization of reactive groups in proteins (8), we think that these data are consistent with the hypothesis that Tos-Phe$CH_2Cl$ inactivation of RaIFN and MuIFN results from reaction with the imidazole ring of a histidine residue.

**Temperature Dependence of Inactivation.** Because temperature dependence of interferon inactivation by Tos-Phe$CH_2Cl$ is consistent with a nucleophilic substitution reaction at the chloromethyl ketone group, we studied the effect of temperature on the inactivation reactions. As shown in Fig. 4, inactivation by Tos-Phe$CH_2Cl$ was slower at ice-bath temperature and 23°C than at 37°C. Similar results have been obtained with MuIFN.

**Protection Against Inactivation by Ligands of Interferons.** One of the criteria for affinity labeling or active-site-directed irreversible inhibition is that ligands that bind reversibly to the site being modified should slow down or block inactivation (5, 6). Therefore, we expected that various ligands of interferon, especially hydrophobic aromatic ligands, should protect the antiviral activity against inactivation by halomethyl ketone de-

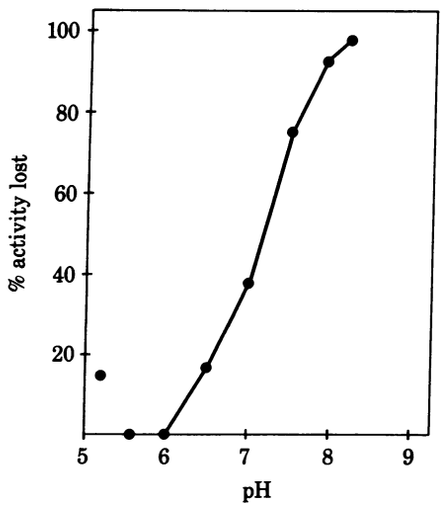


FIG. 3. Effect of pH on inactivation of RaIFN by 1 mM Tos-Phe$CH_2Cl$. RaIFN was in barbital buffer, 0.5 M sodium phosphate buffers were used, and the pH values shown were determined on scaled-up reaction mixtures. Incubation was for 6 hr at 37°C. Results shown were calculated relative to those for control samples run at the same pH values. The final concentration of methanol was 20%.

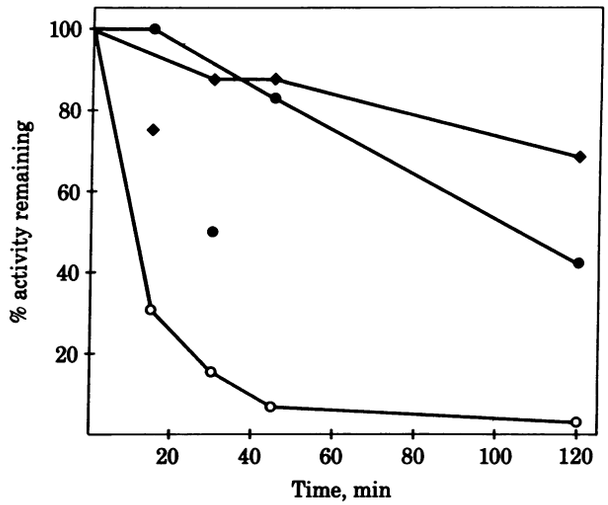


FIG. 4. Effect of temperature on inactivation of RaIFN by 1 mM Tos-Phe$CH_2Cl$. RaIFN was in barbital buffer, 0.5 M sodium phosphate (pH 8) was used, and reaction mixtures contained 20% methanol. Incubation was at 0°C (●), 23°C (◆), or 37°C (○).

rivatives of phenylalanine. Tryptophan and phenylalanine protected RaINF significantly against inactivation by Tos-Phe$CH_2Cl$ (Fig. 5), and a number of derivatives of these aromatic amino acids also protected RaIFN significantly against Tos-Phe$CH_2Cl$ inactivation (unpublished data). This protection did not appear to result from reaction of Tos-Phe$CH_2Cl$ with either the amino or the carboxyl group of the amino acids because derivatives in which these groups are blocked, such as tryptophan methyl ester and *N*-acetyltryptophan, gave similar effects.

Mixed brain gangliosides protected RaIFN completely against Tos-Phe$CH_2Cl$ inactivation, whereas poly(I) was without effect (Table 2). Gangliosides appeared to give more complete protection than tryptophan or phenylalanine. Gangliosides, but not poly(I), were also able to protect MuIFN against inactivation by Tos-Phe$CH_2Cl$ (Table 2).

Bovine serum albumin, another ligand of interferons (9, 10), can block Tos-Phe$CH_2Cl$ inactivation of RaIFN and MuIFN completely (data not shown). However, because bovine serum albumin itself tightly binds hydrophobic compounds, the interpretation of this finding is ambiguous.

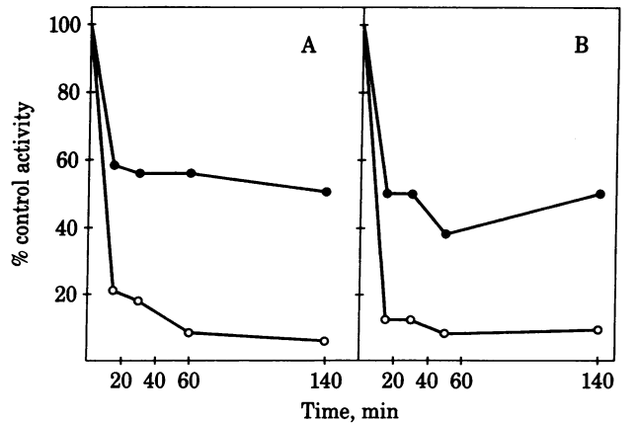


FIG. 5. Effects of aromatic amino acids on the inactivation of RaIFN by 1 mM Tos-Phe$CH_2Cl$. (*A*) Mixtures were incubated with (●) or without (○) 10 mM L-tryptophan. (*B*) Mixtures were incubated with (●) or without (○) 10 mM L-phenylalanine. The RaIFN used was in phosphate-buffered saline/water (1:5), 0.1 M sodium phosphate (pH 8.0) was used, and the final concentration of methanol was 20%.

Table 2. Effects of gangliosides and poly(I) on inactivation of interferons by Tos-PheCH$_2$Cl

| Addition | % control activity | |
|---|---|---|
| | RaIFN* | MuIFN† |
| None | 8 | <4 |
| Gangliosides (1 mg/ml) | 92 | 133 |
| Poly(I) (1 mg/ml) | 8 | <4 |

The buffer was 0.1 M sodium phosphate (pH 8.0), and reaction mixtures contained 1 mM Tos-PheCH$_2$Cl and 20% methanol.
* RaIFN in phosphate-buffered saline/water (1:5); incubation, 130 min; 37°C.
† Incubation, 15 min; 37°C.

**Reaction of Interferon with Serine-Specific Inhibitors.** Our results suggested the possibility that interferons might be chymotrypsin-like enzymes. If this is true, interferons should be inactivated by inhibitors of serine proteases. We have confirmed and extended the results of Merigan *et al.* (11) with chicken interferon by showing that diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride do not inactivate RaIFN either with or without prior incubation at 37°C. In addition, preliminary experiments designed to demonstrate esterase activity in interferon preparations using the 1-naphthyl esters of *N*-carbobenzoxytryptophan and *N*-carbobenzoxyphenylalanine (12) as potential substrates were not successful (unpublished data). These results suggest that interferon is not a serine or thiol protease, although the possibility that it is a different type of highly specific protease cannot be excluded.

## DISCUSSION

We propose that halomethyl ketone derivatives of phenylalanine specifically inactivate interferons by an affinity labeling mechanism. Presumably, these compounds first bind to the interferon through their hydrophobic aromatic rings and then react irreversibly, possibly with a nearby histidine imidazole ring, through their reactive halomethyl ketone groups.

Although direct proof for a specific modification of interferon will require peptide mapping and amino acid analysis of interferons inactivated by halomethyl ketone derivatives of phenylalanine, there is strong, albeit indirect, evidence that such a mechanism underlies the phenomenon we have characterized (6). These arguments are (*i*) the specificity of the inactivation, which is mediated by halomethyl ketone derivatives of phenylalanine but not of lysine; (*ii*) the protection against inactivation conferred by compounds known to bind to a hydrophobic binding site; and (*iii*) the pH dependence of the reaction. The chemical reactivity of the halomethyl ketone group would theoretically permit reaction with any of the different nucleophilic groups in proteins (8). However, of the potentially reactive amino acid side chains, only imidazole and thiol groups have been shown to react with the phenylalanine halomethyl ketones used in these studies (6, 13–16). Thus, rapid modification of cysteine or histidine residues by halomethyl ketones was found to occur in a variety of serine and thiol proteases (17). The specificity of this reaction is most striking in the case of trypsin-like and chymotrypsin-like enzymes in which the substrate-related structure of the alkylating reagent determines the rate of inactivation. A similar type of specificity appears to exist for the inactivation of interferon by halomethyl ketones. Only derivatives of phenylalanine that most likely interact with hydrophobic residues in the target protein cause rapid inactivation; an analogous lysine derivative is without effect, even after prolonged incubation. Therefore, we conclude that the rapid inactivation of the interferons by halomethyl ketones is due to specific interactions of these reagents with a hydrophobic binding site and subsequent modification of an essential amino acid residue.

This interpretation is supported by our protection studies. Tryptophan and phenylalanine protect significantly against inactivation of RaIFN by Tos-PheCH$_2$Cl. These results are consistent with the well-known ability of Sepharose-coupled aromatic ligands to bind to interferons (18).

The complete protection of RaIFN and MuIFN by mixed gangliosides against inactivation by Tos-PheCH$_2$Cl is even more interesting. Gangliosides have been implicated as cellular receptors for binding of interferon to target cells (2), and it is tempting to assume that the binding site of interferon involved in this function is identical with or close to the hydrophobic area interacting with halomethyl ketones. In contrast, poly(I) failed to protect against inactivation. This compound and, to a lesser degree, other single-stranded polynucleotides have been reported to bind to a specific site that overlaps the hydrophobic site responsible for binding of interferon to immobilized Cibacron blue F2GA (19). Whether this reflects an intrinsically lower binding affinity for the polynucleotide or a polynucleotide attachment site different from the hydrophobic site that interacts with the halomethyl ketone is not clear.

The effect of pH on inactivation of the interferons by phenylalanine halomethyl ketones shows that a group or site on the interferon molecule ionizing in the region around neutrality is involved in this reaction. Chymotrypsin and subtilisin, two serine proteases inactivated by phenylalanine halomethyl ketones, show a similar dependence of the reaction with these substances on a group ionizing in the pH 7 region (6, 15). In both cases, the imidazole ring of a histidine residue is the site of reaction (6). By analogy, our results are consistent with the hypothesis that phenylalanine halomethyl ketones react with one or more histidine residues to inactivate interferons. Although we cannot exclude the involvement of a sulfhydryl group with an abnormally low p$K_a$ in the inactivation of the interferons by phenylalanine halomethyl ketones, this appears unlikely because we could not inactivate the interferons with the sulfhydryl reagents iodoacetic acid and bromoacetic acid. This confirms the conclusions of others that sulfhydryl groups are not essential for the antiviral activity of interferons (20). In this context, it is striking that the pH dependence of Tos-PheCH$_2$Cl inactivation of RaIFN is similar to that of inactivation of interferons by ethoxyformic anhydride or dye-sensitized photooxidation. Based on these results, we conclude that interferons could have essential histidine residues (unpublished data).

That halomethyl ketones are excellent probes for defining functionally important sites of proteins has also been shown for several enzymes not related to proteases. Thus, luciferase is inactivated by Tos-PheCH$_2$Cl, but not by Tos-LysCH$_2$Cl, by an affinity labeling mechanism. In this case, two active site sulfhydryl groups are modified by the reagent (21). The catalytic subunit of cAMP-dependent protein kinase is also inactivated by Tos-PheCH$_2$Cl, apparently by an affinity labeling mechanism involving sulfhydryl groups (22). The general implication of these inactivation experiments is that, in the case of phenylalanine halomethyl ketones and related compounds, the modification usually occurs at a hydrophobic binding site that contains reactive histidine or cysteine residues.

Assuming that our hypothesis is correct, we anticipate that it will be possible to isolate a peptide of interferon that has reacted with the phenylalanine halomethyl ketone. Such a peptide would represent a portion of the interferon amino acid sequence located at or very close to the site(s) involved in hydrophobic binding. Because our results suggest that this hydrophobic binding site is essential for the antiviral activity of interferons, we speculate that isolation or synthesis of this se-

quence may lead to an active fragment of the interferon molecule. Tos-Phe$CH_2$Cl- or iodine-labeled derivatives could also be useful if crystallization of interferon is achieved and x-ray crystallography becomes a possibility for studying the three-dimensional structure of the protein.

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