PREFERENTIAL TRITIUM LABELLING OF BINDING SITE RESIDUES IN ALPHA CHYMOTRYPSIN BY EXPOSURE OF THE 1,3-^{3}H-DIISOPROPYLPHOSPHORYL DERIVATIVE TO TRITIATED HYDROGEN SULFIDE

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SUMMARY

When alpha chymotrypsin, labelled with a heavily tritiated diisopropylphosphoryl (^{3}HDIP) group on the serine residue at position 195, was exposed to tritiated hydrogen sulfide (^{3}HSH) under dry conditions, an incorporation of ^{3}H occurred that favored residues close to the binding site. The reaction mechanism remains unclear. A direct transfer of ^{3}H from the ligand is ruled out, although ^{3}H in this position as well as in ^{3}HSH is essential for the labelling reaction. It is shown that the reaction does not involve self-radiolysis. It is suggested that the reaction may instead be related to an observed instability of ^{3}H within the ligand, possibly through a free radical mechanism.

INTRODUCTION

Reaction of carbon free-radicals with tritiated hydrogen sulfide (^{3}HSH) has been employed as a means of studying their distribution among amino acid residues after irradiation of a protein in the lyophilized state at low pressure (1). The present investigation arose from application of this technique to the study of self-radiolysis of an isotopically labelled protein. Chymotrypsin, labelled with a tritiated diisopropylphosphoryl (^{3}HDIP) group, was chosen for the study.

Reaction of chymotrypsin with diisopropylfluorophosphate (DFP) introduces the DIP group onto the hydroxyl oxygen of the serine residue at position 195, the "active site" serine. The reagent was ^{3}H-labelled DFP (^{3}HDFP) with the

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highest specific activity commercially available. The resulting ^{3}HDIP-chymotrypsin was exposed to ^{3}HSH after several weeks of storage. On analysis of the protein, ^{3}H was found among the amino acids, and the distribution of activity favored residues of the kind closest to the binding site (2).

A continued investigation has made possible the differentiation between two effects in the labelling process. The first of these is the expected effect of self-radiolysis. The second involves an interaction between ^{3}HSH and the tritiated ligand-protein complex to introduce additional ^{3}H into the protein. This reaction accounts for at least a part of the preferential labelling of residues near the binding site and is evident without storage of the protein sample.

METHODS

Reaction of Chymotrypsin with ^{3}HDFP

The ^{3}HDFP was obtained from the Amersham-Searle Corporation in propylene glycol with a specific activity of 3.3 Ci/mmole. One mCi of the reagent was added in 3 fold molar excess to a solution of beef pancreatic alpha chymotrypsin (Worthington, crystalline, with an enzymatic activity of 65 U/mg) in 0.05 M tris (hydroxymethyl) aminomethane-HCl buffer of pH 7.4. The mixture was allowed to stand at 30°C for 30 min. and then dialyzed against 4500 ml water at +5°C for 17 hours. The dialysate was changed and dialysis continued against the same volume for an additional 6 hours. The sample was then lyophilized. Specific activity of the product ranged from 30 to 105 μCi/mg.

Exposure of Protein to H_2S and ^{3}HSH

An exposure vessel of the design employed earlier (3) was used in all experiments. The protein samples were exposed to H_2S or ^{3}HSH as already described (1) and then dissolved in 50 ml water and lyophilized. Alternatively some of the samples were exposed to H_2S at 195°K as previously (4).

Determination of ^{3}H in H_2S

After exposure of the protein to H_2S, the gas was drawn into a bulb approximately 1 inch in diameter, containing 2 ml of 1 M NaOH, which had been immersed in liquid N_2. The bulb was allowed to thaw and stirred magnetically

for 15 min. The freezing and thawing were repeated, and the contents dissolved to 100 ml with water in preparation for scintillation counting. For counting, 0.1 ml of this solution was mixed with 15 ml of a liquid scintillation mixture (3a70B, from Research Products International Corp.). A Beckman Model LS-250 scintillation counter was employed.

Isolation of Peptide 193-199

Approximately 10 mg of the ^{3}HDIP-chymotrypsin, which had undergone exposure to ^{3}HSH, was diluted 5 to 6 times by addition of unlabelled DIP-chymotrypsin and then denatured by reduction and carboxymethylation as already described (5). It was chromatographed by the method of Hartley (6) to isolate the C chain which was obtained in yields of 5 to 10 mg. The sample was desalted on a column of Sephadex G-25, equilibrated in 0.1 M acetic acid and was confirmed as C chain by amino acid analysis.

For tryptic digestion, the C chain was redissolved in 3 ml 0.05 M ammonium bicarbonate, and 0.03 ml trypsin (Sigma Chemical Co., 3 x crystallized at 5 mg/ml in water) was added. Digestion was allowed to proceed for 17 hours at 37°C, and the sample was lyophilized.

The procedure of Gross (7) was employed for reaction of the tryptic digest with cyanogen bromide.

For chymotryptic digestion of the cyanogen bromide digest, the sample was redissolved (after lyophilization) in 3 ml of 0.05 M ammonium bicarbonate, and 0.05 ml chymotrypsin (1 mg/ml in water) was added. Digestion proceeded for 17 hours and then a second 0.05 ml of enzyme solution was added. Digestion was continued 6 hours, and the solution was lyophilized.

For separation of peptide 193-199, the chymotryptic digest was subjected to electrophoresis by the method described earlier (8), in pyridine-acetic acid buffer at pH 3.6, with a gradient of 70 v/cm, for 2 hours. The peptide band of maximum count (22 to 24 cm from the origin) was excised, eluted with water, lyophilized, and acid-hydrolyzed. It was then subjected to amino acid analysis and counting as described earlier (9).

RESULTS AND DISCUSSION

A control experiment, conducted in the course of an investigation of the

TABLE I

EXPOSURE OF DIP-CHYMOTRYPSIN[a] TO ^{3}HSH

	Experimental conditions		Specific activity (μCi/mg)		
	Time of exposure (hrs.)	Temperature (°K)	Due to ^{3}H-DIP[b]	Due to ^{3}HSH	
				Exptl.[c]	Calc.[d]
Sample no.					
1	0.5	298	30	0	0
2	6	195	30	0	0.046
3	6	298	30	0.14	0.045
4	6	298	0	0	-
5	6	298	105	0.22	0.10

a. The protein sample was either unlabelled DIP-chymotrypsin or ^{3}H-DIP-chymotrypsin, prepared as described in text.

b. Only the specific activity before exposure to ^{3}HSH was determined. Technical difficulties in removing quantitatively the tritium absorbed on exchangeable sites with the protein (i.e. on O, N, or S) prevented meaningful determination of specific activity after exposure. For this purpose, unlabelled H_2S was used, as indicated in Table II.

c. Experimentally determined specific activity of the protein resulting from exposure of the sample to ^{3}HSH, determined by summation of counts found under peaks.

d. The calculated specific activity, defined as:

$$\frac{S - Sg}{258},$$

where S = specific activity of the protein due to tritium content within the ^{3}H-DIP group before exposure to H_2S (Table II, Column 2), and Sg = specific activity of the protein due to remaining ^{3}H content after exposure to the gas. The constant 1/258 takes into account the ratio of H_2S to ^{3}HSH (1/129) and the effective labelling specific activity of the tritiating gas, which is one half of its measured activity.

self-radiolysis of ^{3}HDIP-chymotrypsin, involved exposure of this derivative to ^{3}HSH with no prior period of storage. Analysis of the protein then revealed a ^{3}H content within the amino acids. It then became necessary to learn more about the ^{3}H transfer in order to distinguish its effects from those of self-radiolysis.

Conditions for ^{3}H Transfer

An exposure time of 6 hours was used for most of the experiments of Table I, since this time interval had been employed routinely in studies of

self-radiolysis and had proven adequate for complete reaction of ^3HSH with the radical content of the protein sample.

Six hours at room temperature (Samples 3 and 5, Table I) resulted in an incorporation of ^3H into the protein (Column 5). On the other hand, 0.5 hour was clearly insufficient for the transfer. The reaction also failed to develop when exposure was carried out for 6 hours at 195°K (Sample 2) and therefore is temperature dependent.

The reaction is also dependent on ^3H content of the DIP group. Sample 5, with a specific activity of 105 μCi/mg, developed an activity of 0.22 μCi/mg for ^3H content within the amino acid residues, whereas Sample 3, with 30 μCi/mg, developed only 0.14 μCi/mg. For Sample 4, in which the protein had reacted with unlabelled DFP, there was no detectable incorporation of ^3H by exposure to ^3HSH.

Exposure of ^3HDIP-Chymotrypsin to H_2S

Since, as seen above, the presence of ^3HSH as the sole contributor of ^3H was insufficient to produce labelling, the reverse situation was examined, i.e. the exposure of ^3HDIP-chymotrypsin to unlabelled H_2S. At best only traces of ^3H accumulated in the protein when it was exposed to H_2S at room temperature for 6 hours. Thus, reaction with ^3HSH is necessary for a significant transfer of ^3H to protein.

However, Table II shows that ^3H within the DIP group is unstable in the presence of H_2S, since substantial quantities of ^3H appeared in the gas phase and the specific activity of the protein markedly diminished. It is also significant that the activity appeared with H_2S, since it was condensed at the temperature of liquid N_2. Tritium gas, on the other hand, does not condense at this temperature.

The loss of ^3H is independent of temperature. Samples 2 and 3, exposed to H_2S in dry ice and at room temperature, respectively, exhibited nearly identical specific activities (Column 3, Table II). The percent of ^3H found in the gas for these samples did not agree as well. However, the gas counting technique should be regarded as only semi-quantitative, and the change in specific activity of the protein is the more reliable indicator of the extent

TABLE II

EXPOSURE OF ^{3}HDIP-CHYMOTRYPSIN TO H_2S

Sample no.[a]	Specific activity[b] (μCi/mg) Before H_2S	After H_2S	^{3}H in gas[c] (% of total)
1	30	30	0
2	30	17.9	42.3
3	30	18.4	18.6
5	105	77.5	26.2

a. Sample numbers correspond to those of Table I.
b. Activity due to presence of ^{3}H in the ^{3}HDIP group.
c. Percent of total ^{3}H activity, originally in the protein sample, appearing in the gas phase.

to which ^{3}H was lost. Moreover, no assurance can be offered that all of the ^{3}H in the gas phase was in the form of ^{3}HSH.

Relationship between ^{3}H Loss and Free-Radical Formation

It appears reasonable to speculate that the instability of the carbon-^{3}H bond may be related to the gain of ^{3}H by the protein. However, a direct transfer is ruled out, since it is already established that ^{3}H transfer in the presence of H_2S does not occur to a significant extent.

The gain of ^{3}H by protein could proceed through a reaction of ^{3}HSH with carbon free-radicals, as discussed previously (1). Hence in search of an explanation for the ^{3}H incorporation it is first necessary to examine the possibility of free-radical formation. Free radicals would unquestionably ensue from absorption of β radiation energy that results from the disintegration of ^{3}H. For a protein sample with a specific activity of 105 μCi/mg, as in Sample 5, there would be, over a 6 hour period, a total of 8.4×10^{10} disintegrations per mg. The energy of the average β particle resulting from disintegration of a ^{3}H atom is 5700 ev (10). With the assumption that the energy associated with production of a free radical is 45 ev (11) and that all the radiation energy is absorbed by the sample and converted to carbon free-radicals, then the

total number of such radicals formed within the sample would be 1.06×10^{13} for 1 mg. It is also assumed that the ^3HSH would react completely with these radicals. However, the isotope effect, allowing for the difference in the reactivities of hydrogen and ^3H is unknown for this system and is therefore neglected. The ^3HSH, with a specific activity of 224 μCi/mmole, would then impart a specific activity to the protein of 0.002 μCi/mg. With a value greater than unity for the isotope effect, this figure would be even smaller. However, an activity approximately 100 times greater was found (Sample 5, Column 5, Table I). For Sample 3, the specific activity would be proportionately less, i.e. 6×10^{-4} μCi/mg, and the experimentally found activity is about 230 times greater. The reaction of ^3HSH with free radicals formed by self-radiolysis therefore could not account for the magnitude of ^3H incorporation, nor could self-radiolysis explain the loss of ^3H into the gas phase.

There is, however, more nearly an equivalence of the leaving ^3H with the extent of reaction with ^3HSH (as indicated by the incorporation of ^3H into the protein). Thus the specific activities due to the incorporation of ^3H (Samples 3 and 6 in Column 5, Table I) agree within factors of 2 to 3 with the corresponding values in Column 6. These results suggest that the loss of ^3H may be related to the reaction with ^3HSH.

Distribution of ^3H

Amino acid analysis (Table III) of the whole protein hydrolysate indicates that those amino acids carrying the most ^3H are of the kind found close to the DIP group. Thus, only ten of the eighteen amino acids of chymotrypsin were labelled to a measurable extent, and this list includes those of the kind present in the vicinity of residue 195 (note content of peptide 193-199, Table III). In addition there is a methionine at position 192 and a half-cystine at position 191; these amino acids also exhibit slight activity.

Isolation and analysis of peptide 193-199 (Table III) confirms that the residues within this area are indeed heavily labelled. The sum of activities for the amino acids of the isolated peptide constitutes 32.2% of the sum for the labelled amino acids in the protein sample from which the peptide was taken,

TABLE III

CONTRIBUTIONS OF AMINO ACIDS TO SPECIFIC ACTIVITIES OF PROTEIN AND PEPTIDE AFTER EXPOSURE OF ^{3}H-DIP-CHYMOTRYPSIN TO ^{3}HSH

	DPM/μmole[a] (x residues x 10^{-6})	
	Protein[c]	Peptide[d]
Aspartic acid	0.48	0
Serine	11.1	2.19
Glutamic acid	0.20	-
Proline	1.10	1.16
Glycine	1.37	1.20
Cystine	0.36	-
Methionine	0.64	-
Leucine	1.33	1.31
Lysine	0.32	-
Arginine	1.26	-
Total ◘	18.16	5.86[e]

a. The specific activity for each amino acid was found by dividing its total counts by efficiency of the counting system (typically 0.015) to obtain DPM. This was divided by the micromoles of amino acid found on analysis.
b. The specific activity was multiplied by the number of residues known to be present within the chymotrypsin molecule (or peptide) for the amino acid in question to obtain a measure of the total contribution of that amino acid to the activity of the protein (or peptide).
c. This sample is No. 5 as shown in Table I.
d. Peptide 193-199, defined as: Gly.asp.ser.gly.gly.pro.leu, is isolated from Sample No. 5 as described in text. For peptide analysis, the actual figures obtained were multiplied by 5.4, since the protein had been diluted by this factor with unlabelled DIP-chymotrypsin.
e. Contribution of peptide to total activity within the protein. Thus, 5.86/18.2 (x 100) = 32.2%.

with 80% of the chymotrypsin molecule accounted for*, despite the fact that this sequence comprises less than 3% of the protein chain length.

* Aspartic and glutamic acids exchange with the medium during acid hydrolysis and consequently lose most of their ^{3}H content. Tyrosine partially exchanges under these conditions (12). Tryptophan could not be included, since the method of analysis employed does not reveal this amino acid to an extent permitting quantitative determination.

Conclusions

The preferential labelling of binding site residues observed after self-radiolysis may therefore be explained at least in part by the transfer of ^3H that occurs on exposure of ^3HDIP-chymotrypsin to ^3HSH with no prior storage. It should now be possible to study self-radiolysis by subtraction of the ^3H content due to the 'zero time' reaction from the total incorporated ^3H resulting from exposure of the ligand-protein complex to ^3HSH after a significant time of storage.

The mechanism of this reaction remains unclear. Direct transfer of ^3H from the ligand is not involved, since tritiation does not occur in the presence of unlabelled H_2S. The approximate equivalence of the leaving ^3H with the reaction of ^3HSH suggests that these two events may be related, possibly through a free-radical mechanism.

Further studies are in progress to shed more light on the nature of this reaction and also to examine the possible application of ^3H labelling to the identification of binding site residues in other proteins.

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REFERENCES

1. White F. H., Jr., Riesz P., and Kon H. - Radiation Res. 32: 744 (1967).
2. White F. H., Jr. - Radiation Res. 51: 458 (1972).
3. White F. H., Jr., Kon H., and Riesz P. - Radiation Res. 45: 8 (1971).
4. Riesz P. and White F. H., Jr. - Radiation Res. 44: 24 (1970).
5. White F. H., Jr. - in Methods in Enzymol., Vol. 25, Part B, p. 387, Ed. by Hirs C.H.W. and Timasheff S. N., Academic Press, Inc., New York, 1964.
6. Hartley B. S. - in Structure and Activity of Enzymes, p. 45. Ed. by Goodwin T. W., Harris J. I., and Hartley B. S. Academic Press, Inc., New York, 1964.
7. Gross E. - in Methods in Enzymol., Vol. 11, p. 238. Ed. by Hirs C. H. W., Academic Press, Inc. New York, 1967.

8. White F. H., Jr. - J. Biol. Chem. 236: 1353 (1961).

9. White F. H., Jr. and Mercken C. R. - Anal.Biochem. 34: 560 (1970).

10. Feinendegen L. E. - Tritium Labelled Molecules in Biology and Medicine. Academic Press, Inc., New York, 1967.

11. Apelgot S. and Duquesne M. - Int. J. Radiation Biol. 7: 65 (1963).

12. Riesz P., White F. H., Jr., and Kon H. - J. Am. Chem. Soc. 88: 872 (1966).