The Chemistry and Identification of im-Dinitrophenyl Histidine*

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SUMMARY

Im-Dinitrophenyl histidine does not decompose under the standard conditions of protein hydrolysis, and it emerges between phenylalanine and lysine on the short column of the amino acid analyzer. Its ninhydrin color constant was found to be 76% of that for histidine. A method for analysis of im-dinitrophenyl histidine in a reacted protein is described. 1-Fluoro-2,4-dinitrobenzene reacts preferentially with the \( \delta \)-nitrogen of the imidazole ring of \( \alpha \)-N-acetylhistidine. The pK of the imidazole ring of im-dinitrophenyl histidine is 3.0 to 3.1.

In the course of chemical modification studies on human carbonic anhydrase B, I found that Sanger's reagent, 1-fluoro-2,4-dinitrobenzene, inactivates this enzyme, reacting rapidly with the imidazole ring of 1 histidine residue (1). I thus became interested in the chemistry and analysis of im-DNP-histidine. A study of the literature revealed conflicting ideas about the stability of im-DNP histidine to protein hydrolysis conditions. While Hirs, Halman, and Kycia (2) maintained that im-DNP histidine decomposed during acid hydrolysis, Siepmann and Zahn (3) reported a synthesis of im-DNP-histidine in which the final step is an acid hydrolysis. However, no quantitative methods of analysis of im-DNP-histidine have been reported. During chemical modification studies on proteins it is often advantageous to be able to analyze for a modified amino acid rather than for the absence of the unmodified residue, since such a decrease may represent only a small percentage of the total quantity of that amino acid in the protein. In this paper I will describe a method for the quantitative analysis of im-DNP-histidine in proteins and will describe several experiments on its chemical properties.

MATERIALS AND METHODS

Chemicals—FDNB, iodoacetic acid, and 2-hydroxy-5-nitrobenzyl bromide were purchased from Mann Research Laboratories. \( \alpha \)-N-Acetylhistidine was obtained from the Sigma Chemical Corporation. Amino Acid Analysis—A Beckman-Spinco amino acid analyzer model 120C with the chromatographic system of Spackman, Stein, and Moore (4) was used. Proteins were hydrolyzed in sealed evacuated tubes containing 6 N HCl for 24 hours at 110°. Synthesis of im-DNP-Histidine—The synthesis of im-DNP-histidine followed the simple route previously taken by Margolis (5) and by Siepmann and Zahn (3).

One gram (4.65 mmoles) of \( \alpha \)-N-acetylhistidine was dissolved in 20 ml of 0.5 M N-ethylmorpholine, and the pH was adjusted to 7.5 with glacial acetic acid. To this, 30 ml of acetone containing 866 mg (4.65 mmoles) of FDNB were added, and the mixture was heated in a round bottom flask with reflux condenser overnight at 60°.

After cooling, the buffer and solvents were removed on a rotary evaporator. The acetyl group of the product, presumably N-ethyl-im-DNP-histidine, was hydrolyzed with 25 ml of 6 N HCl at 105° for 5 hours. During this time the solution became brown. HCl was removed with a rotary evaporator, leaving a brown oil. When 20 ml of glacial acetic acid were added to the product and heated to 100°, a precipitate formed. Cooling and filtering gave light tan crystals weighing 0.5 g after drying (1.46 mmoles, or 32% yield assuming im-DNP-histidine acetate). These crystals were hygroscopic Attempts to recover further crops of crystals were unsuccessful.

The ultraviolet-visible spectrum of the product in 0.1 N HCl was very similar to that shown by Siepmann and Zahn (3). Unlike other \( N \)-substituted DNP derivatives there was no absorption peak above 300 \( \mu \)m. The spectrum showed a steady decline in absorption from 240 \( \mu \)m to 500 \( \mu \)m, with a small shoulder at 340 \( \mu \)m.

RESULTS AND DISCUSSION

Analysis of im-DNP-Histidine in Proteins—When a small amount of im-DNP-histidine was chromatographed on the short column of the amino acid analyzer, a single broad peak appeared just prior to the lysine position, as shown in Fig. 1A. No histidine was present.

This material was shown to be im-DNP-histidine, and not a breakthrough product, by heating it at 80° for 30 min in 0.04 N NaOH. This alkali treatment displaced the DNP group from histidine, regenerating the histidine, as is shown in Fig. 1B. Dinitrophenol was also formed in this reaction and was identified from its ultraviolet-visible spectrum.

In order to determine the stability of im-DNP-histidine to the acid hydrolysis conditions used for proteins, identical ali-
The question naturally arises as to which of these possible terminations of im-DNP-histidine are to be made.

Horton and Koshland (8) have described a reagent, 2-hydroxy-5-nitrobenzyl bromide, which reacts selectively with tryptophan. In experiments carried out in pH 2.2 citrate buffer it was found that a 100-fold excess reagent was necessary to eliminate tryptophan at \(10^{-4}\) M.

The following scheme was used for analysis of im-DNP-histidine and other amino acids in protein hydrolysates. About 30 nmol of protein were hydrolyzed under standard conditions, and the HCl was removed by rotary evaporation. The residue was dissolved in 2.0 ml of citrate buffer, pH 2.2. One milliliter was withdrawn for analysis on the long column, and, to the remaining 1.0 ml, 250 \(\mu\)l of a freshly prepared solution of 25 \(\mu\)mol of HNBB in acetone were added with vigorous swirling. (This is 6.2 pmol of reagent.) After 10 min at room temperature this solution was quantitatively transferred to the 15-cm short column.

Experiments with a standard solution of the amino acids showed that HNBB does not react with im-DNP-histidine but does react with some other amino acid to give a small peak in the position of im-DNP-histidine, such as that seen in Fig. 2B. This corresponds to 5% of 1 residue in carbonic anhydrase B. In analyzing for im-DNP-histidine in a reacted protein, therefore, one must always include a control which consists of an analysis of the unreacted protein treated with HNBB, and the area from this small peak must be subtracted from the DNP-histidine area in the analysis of the reacted protein.


differentiation of the peak is accomplished accurately.

A further difficulty with this method is that tryptophan emerges coincident with im-DNP-histidine on the short column of the amino acid analyzer. Although tryptophan is substantially destroyed by acid hydrolysis, destruction is incomplete and the residual tryptophan must be eliminated if reliable determinations of im-DNP-histidine are to be made.

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Isomers of im-DNP-Histidine—Crestfield, Stein, and Moore (9) have shown that both nitrogen atoms of the imidazole ring of histidine can be carboxymethylated, and that these derivatives are separable on the long column of the amino acid analyzer. Assuming an analogous situation with the dinitrophenylation reaction, there should be two isomers of mono-im-DNP-histidine and also a derivative disubstituted on the ring, 1',3'-di-DNP-histidine.

The question naturally arises as to which of these possible compounds resulted from the synthesis reaction. If more than one molecular species is present, the mixture is not resolved on the column, since there is only one peak (Fig. 1A). On the other
hand, examination of space-filling molecular models leads to the belief that the 3’-DNP-histidine would be favored as there is considerable steric hindrance between the carboxyl group of histidine and the DNP ring in the 1’ isomer. Elucidation of this ambiguity due to isomers is a difficult problem by conventional chemical techniques, and an indirect approach was taken.

This approach was to carboxymethylate the remaining nitrogen of the ring with iodoacetic acid, and then to displace the DNP group with β-mercaptoethanol. If carboxymethylation was complete, this should give rise to 1’- or 3’-carboxymethylhistidine from 3’- or 1’-DNP-histidine, respectively; histidine from 1’, 3’-di-DNP-histidine; and 1’, 3’-dicarboxymethylhistidine for free histidine. (Since the synthetic product was shown to be free of histidine, no 1’, 3’-dicarboxymethylhistidine was expected.) The carboxymethylated derivatives may then be isolated on the amino acid analyzer.

Preliminary experiments showed that carboxymethylation of im-DNP-histidine required vigorous conditions. The im-carboxymethyl-im-DNP-histidine produced was partially resolved from im-DNP-histidine on the short column of the amino acid analyzer, as shown in Fig. 3. Complete carboxymethylation was accomplished by dissolving a mixture of 10.6 mg of α-N-acetyl-im-DNP-histidine (20 μmoles) and 372 mg of iodoacetic acid (2 mmol) in 5 ml of 0.5 M sodium acetate, pH 5.0. This mixture was incubated in a screw cap test tube at 60° for 24 hours. An aliquot was removed, the acetyl group was hydrolyzed, and the sample was chromatographed on the short column of the amino acid analyzer. This showed that more than 90% of the im-DNP-histidine had been carboxymethylated.

The α-N-acetyl-im-carboxymethyl-im-DNP-histidine solution was then incubated with 200 μl (300 μmoles) of β-mercaptoethanol, at 38° for 4 hours, after adjusting the pH to 8.5. An aliquot of 20 μl was removed, hydrolyzed, and chromatographed on the long and short columns of the amino acid analyzer. Only two peaks appeared above background, 89% of the material in a peak at a position corresponding to 1’-carboxymethylhistidine, and 11% as histidine.

I conclude from this result that FDNB reacts predominantly at the 3’-nitrogen in α-N-acetylhistidine, and that little or no 1’-DNP-histidine is formed under these conditions. The presence of a small amount of histidine (11%) in the sample can be more readily attributed to the incomplete carboxymethylation of im-DNP-histidine (see above) than to the presence of im-DNP-histidine.

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