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Use of N^{im}-Dinitrophenylhistidine in the Solid-Phase Synthesis of the Tricosapeptides 124–146 of Human Hemoglobin β Chain*

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ABSTRACT: The tricosapeptide H-Pro-Pro-Val-Gln-Ala-Ala-Tyr-Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Lys-Tyr-His-OH, corresponding to residues 124–146 of the β chain of human hemoglobin, was synthesized by the solid-phase method.

The imidazole rings of the two histidine residues were protected by the dinitrophenyl group and were deprotected by the thiolysis method of Shaltiel. Crystalline N^{α}-*t*-butyloxycarbonyl-N^{im}-2,4-dinitrophenyl-L-histidine was synthesized and shown to couple rapidly and efficiently by the dicyclohexylcarbodiimide procedure. The dinitrophenyl group was stable during the subsequent synthetic steps and in the cleavage step, but could be removed cleanly at the end under mild conditions by treatment with mercaptoethanol. This promises to be a very effective way to handle histidine during solid-phase peptide synthesis.

The C terminus of the β chain of human hemoglobin plays an important part in the determination of the biological activity of this protein. Thus, after the removal of His-146 and Tyr-145 by carboxypeptidase A hemoglobin showed a marked change in its affinity for oxygen and a considerable reduction of the Bohr effect (Rossi-Fanelli *et al.*, 1964). In order to study some of the chemical and physical properties associated with the carboxyl end of the chain we have undertaken the synthesis of the β-chain 124–146 tricosapeptide of human hemoglobin, H-Pro-Pro-Val-Gln-Ala-Ala-Tyr-Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Lys-Tyr-His-OH (Braunitzer *et al.*, 1961).

One of the important problems associated with the synthesis of histidine-containing peptides has been the selection of a suitable protecting group for the imidazole nitrogen of the amino acid. The benzyl group has been used most often for histidine but it does not mask the basicity of the imidazole ring and, in addition, it can be difficult to remove. In cases where catalytic hydrogenolysis is slow or impossible it has been necessary to use sodium in liquid ammonia to remove the benzyl group, and to accept the low yields and side reac-

* From The Rockefeller University, New York, New York 10021. *Received July 11, 1969.* Supported in part by Grant A-1260 from the U. S. Public Health Service and by Grant No. 69,00386 from Consiglio Nazionale delle Ricerche, Italy.

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tions that accompany this method. Synthesis with unprotected, free imidazole has sometimes been successful (Loffet, 1967; Gutte and Merrifield, 1969) but has failed in other instances. The new 2,2,2,-trifluoro-1-benzoxycarbonylaminoethyl (ZTF) group of Weygand *et al.* (1967) is promising, but Boc-L-His-(ZTF)-OH is amorphous and is difficult to obtain pure. In addition, the differential stability of the two groups is marginal. These objections also apply to Boc-L-His(Z)-OH (Ontjes and Anfinsen, 1969). The recent finding by Shaltiel (1967) that the dinitrophenyl group could be removed readily from imidazole by thiolysis led to the introduction by Lombardo, Piazio, and Stewart (cited by Stewart and Young, 1969), of this protecting group for the synthesis of histidine-containing peptides. It has the important advantages of masking the basic properties of the imidazole ring, of considerable stability in acid, and of yielding a Boc-L-His(DNP)-OH derivative that is easily crystallized and is very soluble in organic solvents.

The β-chain tricosapeptide 124–146, which contains two histidine residues, has now been synthesized with the aid of His(DNP) according to the general procedures of solid-phase peptide synthesis (Merrifield, 1963, 1969). The DNP group has permitted the direct esterification of Boc-His(DNP)-OH to chloromethylated resin (Stewart and Young, 1969) without the accompanying quaternization observed with Boc-His(Bzl)-OH (Stewart *et al.*, 1969). The synthesis involved the use of 1 N HCl–HOAc for deprotection of N^{α}-Boc-amino acids, Et_3N–$CHCl_3$ for neutralization, and dicyclohexylcarbodiimide in CH_2Cl_2 for all the coupling reactions except for those involving Gln and Asn, which were with nitrophenyl esters in dimethylformamide. The Boc group was removed from glutamine by trifluoroacetic acid (15 min, 25°) to avoid the possible formation of pyroglutamic acid during the deprotection step (Takashima *et al.*, 1968; Manning, 1968). The other trifunctional Boc-amino acids were Tyr(Bzl) and Lys(Z).

The final peptide was removed from the solid support by HBr in trifluoroacetic acid. This treatment also removed the benzyl from tyrosine and carbobenzoxy from lysine, but left the two His(DNP) residues intact. The DNP was then removed in 1 hr at room temperature by aqueous 2-mercaptoethanol at pH 8. The free peptide was purified by countercurrent distribution. It was then found to be homogeneous on paper electrophoresis at two pH values and gave a good amino acid analysis. The tricosapeptide was digested by trypsin into the three expected peptides, 124–132, 133–144, and 145–146, which were readily separated by electrophoresis at pH 1.9.

On the basis of these experiments the dinitrophenyl group appears to be a very satisfactory protecting group for histidine during solid-phase peptide synthesis. It coupled quantitatively, it was stable during the synthesis and cleavage steps, and it was removed cleanly at the end under mild conditions which do not adversely affect other residues normally used in the synthetic method.

Experimental Section

N^{α}-Boc-N^{im}-DNP-L-Histidine. Boc-L-His-OMe (Schröder and Gibian, 1962; Hofmann *et al.*, 1965) (2.7 g; 10 mmoles) was dissolved in 20 ml of ethanol, and 10 ml of 1 N NaOH was added. After 45 min the solution was neutralized at 0° with 1 N HCl and the ethanol was evaporated *in vacuo*. A solution of 2.3 g of $NaHCO_3$ in 10 ml of water was added, followed by the slow addition of 1.4 ml of 2,4-dinitrofluorobenzene in 15 ml of methanol over a period of 1 hr. The mixture was allowed to react for 10 hr at room temperature. The methanol was evaporated under vacuum, the aqueous phase was washed three times with 20 ml of ether, acidified to pH 3.5 with 1 N HCl at 0°, and extracted three times with 20 ml of ethyl acetate. The extracts were washed with water, dried over $MgSO_4$, and evaporated under vacuum. The oily residue was recrystallized three times from ethanol–petroleum ether (bp 30–60°): yield 2.6 g (62%), mp 94°, $[\alpha]_D^{20}$ +55.3° (*c* 1, ethyl acetate). For analysis, a sample was dried at 58° for 21 hr under high vacuum. *Anal.* Calcd for $C_{17}H_{19}N_5O_8$: C, 48.45; H, 4.55; N, 16.62. Found: C, 48.56; H, 4.81; N, 16.42.

N^{α}-Boc-N^{im}-DNP-L-Histidine-Resin. Boc-His(DNP)-OH (2.9 g, 6.9 mmoles) and triethylamine (0.86 ml, 6.2 mmoles) were dissolved in 30 ml of ethanol and added to 5.3 g of chloromethyl–copolystyrene–2% divinylbenzene beads (1.3 mmoles of Cl/g, 200–400 mesh). The suspension was stirred under reflux for 48 hr, filtered, washed, and dried *in vacuo*, yield 6.1 g. Cleavage of 500 mg of the Boc-His(DNP)-resin with HBr in trifluoroacetic acid for 90 min at 25° gave 65 mg (0.14 mmole) of H-His(DNP)-OH · 2HBr.

Boc-Pro-Pro-Val-Gln-Ala-Ala-Tyr(Bzl)-Gln-Lys(Z)-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His(DNP)-Lys(Z)-Tyr(Bzl)-His(DNP)-Resin. Boc-His(DNP)-resin (2.0 g; 0.56 mmole of His) was placed in the reaction vessel of a manually operated shaker similar to the one described earlier (Merrifield, 1963). The Boc group was removed with 1 N HCl in HOAc for 30 min and the resin was washed three times each with HOAc, EtOH, and $CHCl_3$, neutralized with 10% Et_3N in $CHCl_3$ for 10 min, and washed with $CHCl_3$ and CH_2Cl_2. The resin was then suspended in 4 ml of CH_2Cl_2 containing 928 mg (2.5 mmoles) of Boc-Tyr(Bzl)-OH. After 10 min 2 ml of CH_2Cl_2 containing 515 mg (2.5 mmoles) of *N,N*-dicyclohexylcarbodiimide was added and the coupling reaction was continued for 2 hr. The resulting Boc-Tyr(Bzl)-His(DNP)-resin was washed with CH_2Cl_2 and HOAc and the cycle was repeated 21 more times with the appropriate Boc-amino acid to produce the protected tricosapeptide-resin (3.98 g). Each cycle was the same as described for tyrosine except that Boc-Gln and Boc-Asn were coupled as nitrophenyl esters in dimethylformamide, and the Boc group was removed from Gln by trifluoroacetic acid in place of HCl-HOAc.

H-Pro-Pro-Val-Gln-Ala-Ala-Tyr-Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Lys-Tyr-His-OH (Hemoglobin β Chain 124–146). The protected tricosapeptide-resin (2 g) was washed three times with HOAc and then treated with 10 ml of 1 N HCl in HOAc for 30 min. It was washed with HOAc and ethanol and dried *in vacuo*. The peptide-resin was suspended in 20 ml of trifluoroacetic acid containing 1 ml of anisole and a slow stream of HBr was passed through for 90 min at room temperature. The mixture was filtered and the resin was washed with trifluoroacetic acid. The filtrates were evaporated under vacuum and the residue was extracted with dry ether to yield 203 mg of di-DNP-tricosapeptide.

The peptide was dissolved in 10 ml of H_2O and brought to pH 8 with Na_2CO_3. Mercaptoethanol (2 ml) was added and the pH was readjusted to 8. After 1 hr at 22° the solution was acidified with HOAc and evaporated *in vacuo*.

The product was purified by countercurrent distribution

(130 transfers in the system *sec*-butyl alcohol–1% dichloroacetic acid, 1:1, $K = 0.31$). The contents of tubes 28–34 were evaporated and washed with ether: yield 99 mg, $[\alpha]_D^{18}$ −20° (*c* 1, 80% HOAc). Amino acid ratios were: Asp, 1.06; Glu, 2.02; Pro, 2.06; Gly, 1.01; Ala, 6.07; Val, 3.93; Leu, 0.99; Tyr, 1.90; NH_3, 2.9; Lys, 1.99; His, 1.97.

The product moved as a single spot on paper electrophoresis at pH 5.3 (0.1 M pyridine acetate) with $R_{His} = 0.58$, and at pH 1.9 (formic acid–acetic acid–water, 15:10:75, v/v) with $R_{His} = 0.53$.

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Studies on Lysyl Transfer Ribonucleic Acid Synthetase from *Escherichia coli**

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ABSTRACT: Purification of lysine transfer ribonucleic acid synthetase from *Escherichia coli* grown in ^{32}P-labeled media revealed less than 1 mole of phosphate/mole of enzyme. This ruled out the possibility of oligonucleotides in the recognition mechanism of this enzyme for its cognate transfer ribonucleic acid. No other cofactors were detected in the purified enzyme preparation.

Attempts to preferentially inhibit one of the two activities, the adenosine triphosphate–pyrophosphate exchange and amino acid transfer activity of the apparently homogeneous protein, using inhibitors and limited proteolytic digestion were unsuccessful. Partial protection of both activities in the presence of some substrates was observed. Protection of activities against Nagarse (substilisin) digestion was particularly marked in the presence of transfer ribonucleic acid and lysine. Other properties of the enzyme are presented including amino acid composition, spectral studies, and examination of the inhibition of activity by sulfhydryl reagents.

Aminoacyl-tRNA formation is the first step in protein synthesis. The enzymes which catalyze this reaction, the aminoacyl-tRNA synthetases, must distinguish their specific tRNAs among a large number of very similar RNA molecules. This recognition by an aminoacyl-tRNA synthetase of its specific tRNA is an ideal model for the study of the mechanism of specificity interaction between proteins and nucleic acids. Does the specificity lie entirely in the protein, or are there other groups which participate in the reaction? Coenzymes or metal ions could be invoked, as well as oligonucleotides which would react with the specific tRNA in a manner parallel to the condon–anticodon interaction. No coenzymes or oligonucleotides have yet been determined in aminoacyl-tRNA synthetase reactions. However, it has rarely been possible to obtain sufficient quantities of purified enzymes to assay for these possible enzymatic factors.

The purification and properties of lysyl-tRNA synthetase from kilogram quantities of *Escherichia coli* cell paste have been reported (Stern and Mehler, 1965b; Stern *et al.*, 1966). The purpose of this communication is to demonstrate the absence of stochiometric levels of nucleotides in *E. coli* lysyl-tRNA synthetase, thus ruling out the possibility of oligonucleotides participating in the recognition mechanism between this enzyme and its tRNA.

This investigation also presents additional properties of the

* From the Laboratory of Biochemistry, National Institutes of Dental Research, National Institutes of Health, Bethesda, Maryland 20014. *Received June 16, 1969.* A preliminary report (Stern and Mehler, 1965a) of a portion of the data in this paper was presented at the 49th Annual Meeting of the Federation of American Societies for Experimental Biology, April 1965, Atlantic City, N. J.

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