REEXAMINATION OF DIRECT PREPARATION OF AMINO ACIDS FROM GLYCINE VIA *N*-SALICYLIDENEGLYCINATOAQUOCOPPER (II)

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Received December 28, 1985

Introduction

Glycine can be converted to β-hydroxyamino acids by the formation of metal complex followed by the reaction with carbonyl compound[3,6]. If it is possible to convert glycyl residue in a peptide chain to an another α-amino acid residue, especially to an essential amino acid residue by a simple procedure, an ordinary protein can be changed to a nutritionally excellent protein. Glycylglycine and glycylvaline have been transformed to serylglycine and serylvaline, respectively, by this type of reaction, that is, the formation of complex with Cu(II) followed by the reaction with formaldehyde[5,7]. Triglycine has been changed to threonylgylcyclglycine by the same procedure[2]. The reaction of activated methylene group of glycine with carbonyl compounds, however, always results in the formation of β-hydroxyamino acids. NAKAHARA *et al.* presented in their brief report that α-amino acids are obtained by the reaction of *N*-salicylideneglycinatoaquocopper (II) (*N*-sal-gly-Cu) with alkyl halides[4]. We tried to convert glycine to some amino acids by their method as the first step of the attempt to transform glycyl residue in a peptide chain to some α-amino acid residue, but the yield of each product was far below the reported value and several undesired products, which are not described in the paper cited above[4], were formed. An unexpected formation of diglycine was also observed.

Materials and Methods

General method. Paper chromatography (PC): n-BuOH-AcOH-H_2O (4 : 1 : 2) (solvent 1) and PhOH-H_2O-conc. NH_4OH (120 : 30 : 1. w/v/v) (solvent 2); amino acid analysis: Model 835 Hitachi high speed amino acid analyzer,

Hitachi custum ion-exchange resin #2619 (2.6 mm i. d. × 250 mm), MCI® Buffer 835-PF-Kit under the conditions for physiological fluids analysis.

Preparation of N-sal-gly-Cu. This complex was prepared according to the method of HARADA and OHASHI[3] starting from glycine (0.1 mole), salicylaldehyde (0.1 mole) and cupric acetate (0.1 mole). Yield : 48.3%. The contents of glycine and salicylaldehyde moieties in the complex were determined after acid hydrolysis with an amino acid analyzer and UV spectrometer (OD at 372 nm), respectively. Copper content was measured by an atomic absorption spectrophotometer.

Anal. Found (theoretical value in parentheses) : glycine, 0.8 (1.0) ; salicylaldehyde, 0.9 (1.0) ; Cu, 1.0 (1.0).

Reaction of N-sal-gly-Cu with various kinds of alkyl halides.

1. In dimethylformamide (DMF) with KOH. The complex (1 mmole each) prepared above was reacted with methyl iodide (10 mmole), isopropyl bromide (14 mmole) and isobutyl bromide (10 mmole), respectively, in the presence of KOH (4.5 mmol each) in DMF (20 ml) at 65°C for 3 hours. The amount of alkyl halide and KOH were lowered than the reported values[4], for an enormous amount of KOH used in the reference[4] did not dissolve in the solvent. The reaction mixture was acidified with 6 N HCl in an ice bath. Copper was removed by introduction of H_2S gas. EtOH was added to the filtrate to precipitate NaCl. The filtrate was applied on a column of Dowex 50 (H^+, 2 × 10 cm), which was washed thoroughly with H_2O (*ca.* 1 l). The amino acid fraction was eluted with 2N NH_4OH and analyzed with an amino acid analyzer. The products and their molar ratio are summarized in Tables 1-3.

2. In dimethylsulfoxide (DMSO) with NaOEt. In the reaction mixture of the reaction 1 described above, a large amount of dimethylamine was present as a result of hydrolysis of DMF as mentioned below. Therefore, DMSO and NaOEt were used in place of DMF and KOH. But DMSO was not a suitable solvent for this type of reaction as described later[1]. *N*-Sal-gly-Cu (1 mmole each) was reacted with 1 mmole each of isopropyl bromide, isopropyl chloride, benzyl chloride and 2-chloroethylmethyl sulfide in the presence of NaOEt (10 mmole each) in DMSO (20 ml each) at 65°C for 3 hours. The reaction mixture was treated with the same procedure as described in reaction 1, but the filtrate after H_2S introduction was applied on a cation exchange resin column without addition of EtOH. The products and their molar ratio are summarized in Tables 4-6.

The products of reaction 1. The reaction mixture of *N*-sal-gly-Cu with methyl iodide gave several peaks on an amino acid analyzer (Table 1). Sar-

cosine, unchanged glycine, and alanine were identified by comparison of their chromatographic character on PC and an analyzer with authentic samples. α-Aminoisobutyric acid, which was a main product, was isolated by preparative PC (solvent 1) and identified with NMR and IR spectra. NMR $\delta_{DSS}^{D_2O}$: 1.48(s). IR spectrum (KBr) and chromatographic behavior were consistent with an authentic specimen. Dimethylamine, which was arised from DMF, was isolated as HCl salt and confirmed by comparison of IR spectrum (KBr) with an authentic sample.

The reaction mixture of *N*-sal-gly-Cu with isopropyl bromide gave three peaks corresponding to glycine. glycine ethyl and isopropyl ester and a small peak of valine on an analyzer (Table 2). Each product was identified by comparison of the behavior on PC and an analyzer with authentic sample. Dimethylamine existed in the reaction mixture in quantity, although its peak on the analyzer was small because of the low color yield with ninhydrin. When the reaction mixture was treated with Dowex 50 (NH_4^+) and the same resin of H^+ type successively to isolate basic reaction products, ethyl and isopropyl esters of glycine disappeared and new peaks corresponding to diglycine and glycine amide appeared in the eluate from the column with 2N NH_4OH. The formation of glycine amide and diglycine from glycine ester during a treatment with ion exchange resin of H^+ type was confirmed as follows. Isopropyl ester of glycine was prepared by the conventional method, that is, suspension in isopropanol, introduction of HCl gas and reflux for 1.5 hours. Yield: 93.1%. NMR $\delta_{TMS}^{CDCl_3}$: 1.24 (*d*, J=6 Hz, 6 H), 3.94 (*brs*, 2 H), 5.09 (*dq*. J=6 Hz, 1 H), 8.50 (*brs*, 3 H). Isopropyl ester (0.7 mmole) was dissolved in H_2O and placed on a column of Dowex 50 (H^+, 1 ml). After washed with H_2O (10 ml), the column was eluted with 2 N NH_4OH. The eluate gave three peaks on an analyzer corresponding to glycine, diglycine and glycine amide with the ratio of 24.2 : 0.3 : 1.0. The reaction mixture was put on a column of Dowex 50 (NH_4^+). Neutal fraction containing glycine and diglycine was washed out with H_2O. Glycine amide was eluted from the column with 2 N NH_4OH and identified with FDMS spectrum. FDMS *m/z* (rel. int.): 75 $[M+1]^+$ (84.1), 74 (100). The presence of diglycine in the neutral fraction was also confirmed by FDMS spectrum of the effluent. FDMS *m/z* of fragments beyond *m/z* 80 (rel. int.): 133 $[M+1]^+$ (87.4), 115 [cyclo (Gly-Gly)+1]$^+$ (75.1), 114 (100). The same products were obtained starting from glycine ethyl ester instead of isopropoyl ester, but the ratio of the products was different (2.7 : 0.8 : 1.0 for glycine, diglycine and glycine amide). Glycine amide and a small amount of diglycine were also formed merely by dissolving glycine ester in conc. NH_4OH. The solution gave three

peaks of glycine, diglycine and glycine amide on an analyzer with the ratio of 0.7 : 0.02 : 1.0. Glycine ester disappeared completely. However, dileucine could not be detected in the solution of leucine ethyl ester in conc. NH_4OH. Only two peaks of leucine and leucine amide were recognized on an analyzer. FDMS of isolated leucine amide *m/z* (rel. int.) : 131 $[M+1]^+$ (54.4). Yield : 64.7%. Dimethionine also could not be detected in the solution of methionine ethyl ester in conc. NH_4OH. Main product was methionine amide. FDMS *m/z* (rel. int.) : 149 $[M+1]^+$ (17.3), 148 (100). Yield : 73.6%.

Dimethylamine cristallized out of the reaction mixture of *N*-sal-gly-Cu and isobutyl bromide as HCl salt. The mother liquor which gave two major peaks corresponding to glycine and its ethyl ester and a very small peak of leucine on an analyzer, but did not contain diglycine and glycine amide, was applied on a column of Dowex 50 (H^+). The eluate from the column with 2 N NH_4OH contained diglycine and glycine amide. Glycine ester disappeared completely (Table 3).

The products of reaction 2. The reaction mixture of *N*-sal-gly-Cu with isopropyl bromide in DMSO in the presence of NaOEt gave a big peak of glycine and two modest peaks of valine and serine on an analyzer. Serine was identified tentatively on the basis of the retention time. Although the yield of valine was improved by this method, the amount obtained was still very low (Table 4). When the same reaction was carried out with isopropyl chloride in place of bromide, valine was not formed.

The reaction mixture of *N*-sal-gly-Cu and benzyl chloride contained glycine and β-phenylserine, which were eluted as one peak because of their nearly the same retention time on an analyzer, and a very small peak of phenylalanine (Table 5). β-Phenylserine, which was an unexpected product, was isolated by preparative PC with solvent 1 and identified with NMR and FDMS spectra. NMR $\delta^{D_2O}_{DSS}$: 3.93 (*d*, $J=4.4$ Hz), 4.10 (*d*, $J=4.1$ Hz), 5.34 (2×*d*, overlapped), 7.43 (*s*), 7.47 (*s*). FDMS *m/z* (rel. int.) : 182 $[M+1]^+$ (48.3), 107 (100). These data showed that the product was a mixture of diastereoisomers. It is considered that the phenylalanine derivative was formed from *N*-sal-gly-Cu and benzaldehyde produced from benzyl chloride during the reation. Chromatographic character and NMR spectrum of this product were consistent with a mixture of diasteroisomers of β-phenylserine prepared from glycine and benzaldehyde.

No trace of methionine was detected in the reaction mixture of *N*-sal-gly-Cu and 2-chloroethylmethyl sulfide, but, similarly as above, β-hydroxy derivative was obtained, which was isolated by preparative PC (solvent 1) and identified with NMR and FDMS spectra (Table 6). NMR $\delta^{D_2O}_{DSS}$: 2.14 (*s*),

2.16 (*s*), 2.74 (*m*), 3.83 (*d*, $J=4$ Hz), 3.95 (*d*, $J=4$ Hz), 4.27 (poor resolution). FDMS *m/z* (rel. int.): 166 $[M+1]^+$ (100). It is likely that the β-hydroxy derivative was formed in a similar manner as for β-phenylserine, that is, from *N*-sal-gly-Cu and H_3CSCH_2CHO generated during the reaction.

Results and Discussion

The products obtained by this experiment described above and their yields are given in Tables 1-6. The yield of desired products in reaction 1 was always far below the reported value[4]. But two major products formed by the reaction of *N*-sal-gly-Cu and methyl iodide were α-aminoisobutyric acid and sarcosine and the yield of these two glycine derivatives was far higher than the desired product, alanine (Table 1). Moreover, a large amount of dimethylamine was always formed as a result of hydrolysis of the solvent (DMF) (Tables 1-3). Dimethylamine thus formed is likely to react with

TABLE 1. Reaction Rroducts of *N*-salicylideneglycinatoaquocopper (II) and Methyl iodide (Reaction I*)

Products	Molar Ratio	Identification Method**
Glycine	1.00	PC, AAA
Alanine	0.02	PC, AAA
Sarcosine	0.07	PC, AAA
α-Aminoisobutyric acid	0.14	NMR, IR, AAA
Glycine ethylester	0.03	PC, AAA
Dimethylamine	l.a.***	IR as HCl salt

* See text for reaction conditions.
** PC, paper chromatography; AAA, amino acid analyzer.
*** Large amount.

TABLE 2. Reaction Products of *N*-salicylideneglycinatoaquocopper (II) and Isopropyl bromide (Reaction I*)

Products	Molar Ratio	Identification Method*
Glycine	1.00	PC, AAA
Valine	0.01	PC, AAA
Glycine ethylester	0.58	PC, AAA
Glycine isopropylester	0.09	PC, AAA
Dimethylamine	l.a.*	IR as HCl salt

* See the footnotes in Table 1.

TABLE 3. Reaction Products of *N*-salicylideneglycinatoaquocopper (II) and Isobutyl bromide (Reaction I*)

Products	Molar Ratio		Identification Method*
	Treatment with Dowex 50(H^+)		
	Before	After	
Glycine	1.00	1.00	PC, AAA
Leucine	0.002	0.003	PC, AAA
Diglycine	0	0.003	PC, AAA
Glycine amide	0	0.06	PC, AAA
Glycine ethylester	0.22	0	PC, AAA
Dimethylamine	l.a.*		IR as HCl salt

* See the footnotes in Table 1.

TABLE 4. Reaction Products of *N*-salicylideneglycinatoaquocopper (II) and Isopropyl bromide (Reaction II*)

Products	Molar Ratio	Identification Method*
Glycine	1.00	PC, AAA
Valine	0.02	PC, AAA
Serine	0.03	AAA (tentative)

* See the footnotes in Table 1.

TABLE 5. Reaction Products of *N*-salicylideneglycinatoaquocopper (II) and Benzyl chloride (Reaction II*)

Products	Molar Ratio	Identification Method*
Glycine	not separated. 1.00 for a combined peak	PC
β-Phenylserine		NMR, FDMS, PC
Phenylalanine	0.01	PC, AAA

* See the footnotes in Table 1.

TABLE 6. Reaction Products of *N*-salicylideneglycinatoaquocopper (II) and 2-chloroethylmethyl sulfide (Reaction II*)

Products	Molar Ratio	Identification Method*
Glycine	1.0	PC, AAA
β-Hydroxymethionine	0.3	NMR, FDMS
" (diastereoisomer)	0.5	
Methionine	0	

* See the footnotes in Table 1.

alkyl halide giving a tertiary amine, which can not be detected on PC and an analyzer with ninhydrin reagent.

The formation of these undesired products has not been described in the report cited above[4]. The reasons of lower yield of desired products in this experiment than the reported value[4] are still obscure. The yield of valine increased when NaOEt and DMSO were used instead of KOH and DMF (Tables 2 and 4), but the yield was still very low. Moreover, it was shown that DMSO is also an unsuitable solvent for this type of reaction, because alkyl halide is oxidized to aldehyde or ketone by DMSO[1] as shown by the formation of fairly a large amount of β-phenylserine or β-hydroxymethionine from *N*-sal-gly-Cu and bynzyl chloride or 2-chloroethylmethyl sulfide, respectively, in DMSO (Tables 5 and 6).

An unexpected formation of a small amount of diglycine during reaction 1 is interpreted as follows: Glycine ethylester is formed during the treatment of glycine solution in aqueous EtOH with Dowex 50 (H^+). Thus, the presence of a large amount of glycine ester in the reaction mixture is explained (Tables 1–3). When the reaction mixture containing glycine ester is applied on Dowex 50 (H^+) again and eluted with 2 N NH_4OH, glycine ethylester changes to glycine, glycine amide and diglycine. Diglycine was also obtained merely by dissolving glycine ester in conc. NH_4OH, although the yield was very low. Dileucine or dimethionine, however, could not be detected in the solution of ethylester of leucine or methionine in conc. NH_4OH. The only one product except leucine or methionine was leucine amide or methionine amide, respectively.

The conversion of N-terminal glycyl residue to some β-hydroxyamino acid residue seems to be possible because of the high reactivity of alkyl aldehyde, but the results obtained by this experiment suggest that more complete activation of glycyl methylene group and protection of amino group than in *N*-sal-gly-Cu is required to convert glycyl residue to an α-amino acid residue by the reaction with alkyl halide.

Summary

1. *N*-Salicylideneglycinatoaquocopper (II) (*N*-sal-gly-Cu) was reacted with several alkyl halides to convert glycine to α-amino acid, but the yields were always very low. For example, when methyl iodide was used as an alkayl halide, the yield of α-aminoisobutyric acid and sarcosine, which were undesired products, were far higher than the desired amino acid, alanine. It appears that more complete activation of glycyl methylene group and protection of amino group than in *N*-sal-gly-Cu is required to convert glycyl residue to

an α-amino acid residue by the reaction with alkyl halide.

2. A small amount of diglycine was formed by the treatment of glycine solution in aqueous EtOH with Dowex 50 (H^+). Diglycine was also formed merely by dissolving glycine ester in conc. NH_4OH. Dileucine or dimethionine was, however, not detected in the solution of leucine ester or methionine ester in conc. NH_4OH.

Acknowledgement

We express our gratitude to Mr. K. WATANABE for measurements of the FDMS spectra.

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