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Citation	大阪大学, 1982, 博士論文
Version Type	VoR
URL	https://hdl.handle.net/11094/24344
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Isotope Labeling of the C-2 Atoms of Indoles. Application to Tryptophan-62 in Hen Egg-white Lysozyme

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1982

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PREFACE

It is known that numerous stereo-specific reactions involving a variety of macromolecules are essential to maintain the life of living organisms. These reactions include interactions between nucleic acids and proteins , antigen-antibody reactions, and ligand recognition of receptor proteins in biomembranes.

It is obvious that proteins play essential roles in these interactions as well as in various biochemical reactions. The tryptophan residue has been often found in, or in the vicinity of, the interaction site of proteins probably because this aromatic residue has the hydrophobic indole nucleus which has the big planar structure.

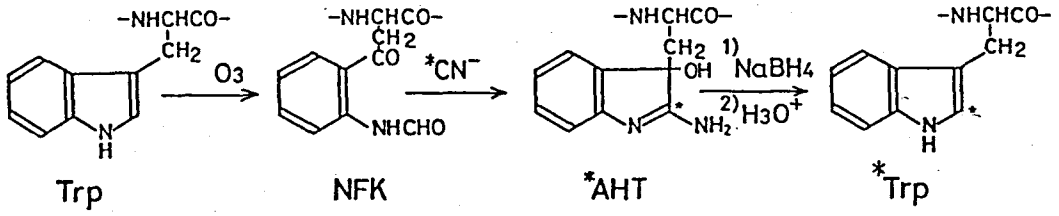
In addition to proteins containing tryptophan, there are many indole derivatives in naturally occurring bioactive substances with low molecular weights. Indole alkaloids and many toxic and hormonal peptides, such as amanitins, adrenocorticotropic hormone, etc, contain the indole nucleus which is essential to their biological function. It appears that the indole nucleus is participated directly or indirectly in the interaction of these substances with their specific targets, which are usually proteins. Accordingly, it is reasonable to postulate that the indole nucleus takes important parts in the recognition and the binding of the ligand molecule in biologically active substances.

Indole itself can not be involved in ionic interaction owing to the lack of ionizable group in physiological conditions.

Weak interactions such as the van der Waals forces and hydrogen bonding at the N-1 nitrogen are possible.

The investigation concerning these weak interactions in which the tryptophan residue is involved has been conducted by X-ray crystallography and by a variety of spectroscopic techniques. High-resolution nuclear magnetic resonance (NMR) spectroscopy has also been applied for proteins in solution, but it is not easy to assign the signals to individual paramagnetic atoms. If the assignment is achieved correctly by some other methods, this technique is very useful to collect information concerning the local structure of a protein molecule in dynamic state. For this purpose, several methods have been devised to introduce an isotopic probe into the specific position of a protein (1, 2).

Recently, a series of reactions have been devised to interconvert between 3-alkylindoles and N-formylanthraniloyl compounds (3, Scheme 1).



Scheme 1.

By this method, the indole C-2 atoms are eventually replaced by the atoms derived from cyanide and borohydride. This fact indicates that the ^{13}C-labeled indole is obtained when the ^{13}C-labeled cyanide is used. Several indole compounds including small trypto-

phan have thus been labeled with ^{13}C at the indole C-2 position.

Then I have undertaken the application of this labeling method to the tryptophan residue of proteins in order to use the enriched ^{13}C atom as a probe for environment analysis of this aromatic residue.

Chapter 1 describes the results of experiments which have been carried out using small indole compounds in order to establish the milder conditions to convert N'-formylkynurenine (NFK) to the corresponding 2-amino-3-hydroxytryptophan (AHT) derivative and to reduce the latter compound with borohydride. Mechanisms are proposed for the formation of AHT and subsequent regeneration of the 1H-indole nucleus. In Chapter 2, the preparation of [2-^{13}C]Trp-62-lysozyme, in which Trp-62 is specifically labeled with ^{13}C at the indole C-2 position, is described. The analysis of the local environment of Trp-62 by ^{13}C-NMR spectroscopy using the incorporated ^{13}C atom as the probe is also described. This Chapter also includes discussions of the role of Trp-62 in the catalytic function of hen egg-white lysozyme.

CHAPTER 1

Reaction Conditions and Mechanisms for the Isotope Labeling of Indole C-2 Atoms

INTRODUCTION

In 1979, a novel method for the reversible chemical conversion between 3-alkylindole and N-formylanthraniloyl derivative was established in our laboratory (3). As shown in Scheme 1, the carbon and hydrogen atoms at the indole C-2 position were eventually replaced by one or both of those atoms derived from the reagents, cyanide and borohydride. Thus, it is possible to use this method for the isotope labeling of indoles.

Although a series of reactions are performed under relatively mild conditions, there is need for improvement in the conditions in order to apply each reaction to chemically labile molecules such as peptides and proteins.

This chapter deals with the reaction of N-formylanthraniloyl compounds with cyanide in aqueous and non-aqueous media.

Reduction conditions of 2-amino-3H-indol-3-ol with sodium borohydride in mM concentrations are also described.

Additionally this chapter includes the ^{13}C-labeling of several small indole compounds by the method established by the present study.

MATERIALS AND METHODS

[1] Starting indole and N-acylanthraniloyl compounds

Indole, skatole (3-methylindole), tryptamine [3-(2-aminoethyl)indole], and 3-(indole-3-yl)propionic acid were purchased from Nakarai Chemicals Co., Kyoto. L-Tryptophan was obtained from Sankyo Kasei Co., Tokyo. Melatonin (N-[2-(5-methoxyindole-3-yl)ethyl]acetamide) was purchased from Sigma (U.S.A.). N^{α}-Acetyl-L-tryptophan (Ac-Trp-OH, mp. 189°C, $[\alpha]_D^{23°C}$ = +28.0°. c= 1, equivalent NaOH) was prepared from L-tryptophan by the method of Warnell et al. (4). N^{α}-Acetyl-L-tryptophan amide (Ac-Trp-NH_2, mp. 195-196°C, lit (5) 192-193°C) was prepared from Ac-Trp-OH by esterification and amidation. 1,2,3,4-Tetrahydrocarbozole (mp. 145-146°C) was prepared by the Fischer indolization reaction from cyclohexanone and phenylhydrazine (6). o-Trifluoroacetylaminoacetophenone (mp. 113-114°C, M^+=231) was a trifluoroacetylation product of o-aminoacetophenone with trifluoroacetic anhydride in trifluoroacetic acid and recrystallized from benzene. Methyl N-formylanthranylate (mp. 35-36°C, M^+=231) was prepared by the reaction of methyl anthranylate with acetic anhydride in formic acid.

[2] Reagents and solvents

Dicyclohexylcarbodiimide (DCC) was obtained from Protein Research Foundation (Osaka) and purified by vacuum distillation (bp. 115-120°C, 4 mmHg). Absolute methanol used as a solvent for ozone-oxidation was dehydrated by boiling over

magnesium turnings and then distilled (bp. 64°C). Other chemicals were of reagent grade and used without further purifications .

[3] Isotopically labeled compounds

Potassium cyanide-^{13}C ($K^{13}CN$, 90% ^{13}C-enriched) was purchased from Prochem (England). Deuterated solvents for NMR measurements: deuterium oxide (99.8% D_2O), dimethyl-sulfoxide-d_6 (99.8% $(CD_3)_2SO$), and chloroform-d_1 (99.8% $CDCl_3$) were obtained from Commissariat A L' énergie Atomique (CEA), France.

[4] Spectroscopic measurements

Ultraviolet (UV) absorption spectra were recorded on a Hitachi Recording Spectrophotometer EPS-3T and infra-red (IR) spectra on a JASCO Infra-red Spectrophotometer IR-G for samples in KBr discs. The electron-impact mass spectra (EI-MS) were taken with a Hitachi RM 50 GC-Mass spectrometer attached with a direct sample-inlet system for solid specimens. Nuclear magnetic resonance (NMR) spectroscopy was performed with a JEOL FX-100 and a FX-200 FT-NMR spectrometers operating at 99.60 and 199.50 MHz respectively for ^1H-NMR measurements, and at 25.05 and 50.10 MHz respectively for ^{13}C-NMR measurements. In both cases, fields were locked on a deuterium signal of the solvents.

[5] Ozone-oxidation of indoles

N-Formylkynurenine (NFK) and its analogs were prepared by the ozonolysis of the corresponding indole compounds

according to the method of Masuda et al (7). A typical ozonolysis experiment is described for Ac-Trp-NH_2.

Ac-Trp-NH_2 (491 mg, 2 mmol) was dissolved in absolute methanol (60 ml) and the solution was cooled in an acetone dry-ice bath (-78°C). To this cold solution was passed a stream of ozone (20-40 µmol O_3/200 ml/O_2/min), which was generated from oxygen with an ozonizer (Nihon Ozone Co., Tokyo, model 0-3-2). The oxidation was followed by the increase and the decrease of absorbance at 360 and 280 nm, respectively. After the disappearence of a peak at 280 nm, dimethylsulfide (0.5 ml, 7.3 mmol) was added to reduce the intermediate N-methoxy-hydroperoxide formed and kept at -78°C for 15 min. Crude crystals of N^{α}-acetyl-L-kynurenine amide (Ac-NFK-NH_2, 515 mg, 93%) were obtained on evaporation of the solvent and the excess reductant under reduced pressure. Recrystallization from methanol gave colorless thin plates, mp. 188-189°C (lit.[7] 189-191°C)

[6] 2-Amino-3-methyl-3H-indol-3-ol

This compound was prepared by three different ways.

i) From 1-formamidoacetophenone

a) In aqueous media: o-Formamidoacetophenone (N-2-acetylphenyl)-formamide, mp. 75-76°C (lit.[8] 78-79°C)) was prepared either by the ozonolysis of 3-methylindole or by formylation of o-amino-acetophenone with formic acid and acetic anhydride.

Well pulverized crystals of o-formamidoacetophenone (1.28 g, 8.0 mmol) were suspended in aqueous solution of potassium

cyanide (550 mg, 8.5 mmol / 5 ml H_2O). The mixture was stirred vigorously overnight in an ice bath. As the reaction proceeded, the insoluble material gradually changed its composition from the starting material to the end product, 2-amino-3-methyl-3H-indole-3-ol, which was filtered, washed with water and then with cold ethanol. Recrystallization from hot methanol gave 940 mg (75%) of colorless granules, mp. 198-200°C (dec.). Anal. Calcd. for $C_9H_{10}N_2O$: C, 66.65; H, 6.62; N, 17.27. Found : C, 16.47; H, 6.14; N, 17.05. MS (m/e) : 162 (95.4%, M^+), 147 (65%, $M^+ - CH_3$), 43 (100%, CH_3CO^+). UV : $\lambda_{max}^{H_2O}$ (ε), 261.5(5380):(pH < 5); 270(8300), 280(6580), 305(2790):(pH > 10). ^{1}H-NMR : (DMSO-d_6) δ(ppm) : 1.38 (s,3H, CH_3), 5.71 (s, broad, 1H, OH), 6.70-7.19 (m, aromatic H and NH_2). ^{13}C-NMR (D_2O) : 175.52 (cation) and 178.72 (non-protonated) for C-2 carbon (pKa : 7.76 ± 0.06).

b) In non-aqueous media : To the methanol solution of o-formamidoacetophenone (640 mg, 4 mmol/5ml methanol) was added solid potassium cyanide (267 mg, 4.1 mmol) and L-proline (460 mg, 4 mmol). In a few minutes of stirring, the mixture became clear and then began to separate out colorless crystalline product. 2-Amino-3-methyl-3H-indol-3-ol , collected by filtration after standing several hours in a cold place was 420mg (65%). This compound was identical with the material obtained in (i).

ii) From o-acetamidoacetophenone in aqueous glycine solution

o-Acetamidoacetophenone, mp. 73.5-74.5°C (lit.(9) 76-77°C), was obtained by acetylation of o-aminoacetophenone with acetic anhydride in aqueous sodium acetate.

Potassium cyanide (78 mg, 1.2 mmol), o-acetoamidoacetophenone (177 mg, 1 mmol) and glycine (75 mg, 1 mmol) were dissolved in aqueous methanol (1 : 1 v/v, 3 ml) with gentle warming. The solution was stirred for one day at room temperature. Cream-colored fine crystals precipitated were collected by filtration, washed with water and recrystallized as described previously. The product (84 mg, 52%) thus obtained was identified to be 2-amino-3-methyl-3H-indol-3-ol from melting point, UV absorption and mass spectra. The filtrate was concentrated to dryness. After removal of o-acetamidoacetophenone (40 mg) by extraction with ethyl acetate, the residue was dissolved in 1M HCl (1 ml) and extracted with ethyl acetate. By evaporation of the solvent, acetylglycine [mp. 204-205°C, (lit. (10) 206°C = aceturic acid) after recrystallization from aqueous ethanol] was obtained.

[7] N^{α}-Acetyl-β-(2-amino-3-hydroxy-3H-indol-3-yl)alanine (Ac-AHT-OH) diastereoisomers from Ac-Trp-OH.

Ac-Trp-OH (492 mg, 2 mmol) was neutralized with potassium bicarbonate (200 mg, 2 mmol) and then ozone-oxidized as described previously. After reduction with dimethylsulfide (0.5 ml), the solvent was replaced by water (3 ml). To this aqueous solution containing potassium salt of N^{α}-acetyl-N'-for-

myl-L-kynurenine (Ac-NFK-OH) were added potassium cyanide (130 mg, 2 mmol) and methylamine hydrochloride (130 mg, 2 mmol). The reaction mixture was stirred for 10 hours at room temperature, concentrated to about 2ml after the addition of 1M HCl (2 ml, 2 mmol), and allowed to stand overnight. Thin plates of a less water soluble diastereoisomer of Ac-AHT-OH were collected and washed with cold water and ethanol, successively. The yield was 220 mg (40%), mp. 237-238°C (dec.). $[\alpha]_D^{18°C}$ - 37.6° (c 0.5, 0.1M HCl). Anal. Calcd. for $C_{13}H_{15}N_3O_4$: C, 56.31; H, 5.45; N, 15.16. Found : C, 56.23; H, 5.39; N, 15.13. IR (KBr): 3210, 1675, 1650, 1622 and 1193 cm^{-1}. UV (λ_{max}/nm (ε)) : pH<5, 265 (3790) and 297 (2870); pH>10, 274 (6590), 284 (5040) and 311 (2570). ^{1}H-NMR (DMSO-d_6, with a few drops of 1M ^{2}HCl) : 1.45 (s, 3H, $COCH_3$), 2.56-2.65 (m, 2H, β-CH_2), 3.99 (d.d., J=3.7 and 8.2 Hz, 1H, α-CH), 7.09-7.51 (m, 4H, arom.H), and 7.90 (d, J=8.2 Hz, unexchanged amide NH). Crystals of Ac-AHT-OH for X-ray diffraction experiments were prepared by careful neutralization of the 1M HCl solution with NaOH. The absolute configuration of this material was determined to be 2S,3R by X-ray crystallographic analysis. The analysis was performed by Dr. Hata et al (11).

The filtrate was concentrated to dryness under reduced pressure. After the removal of inorganic salt by the addition of methanol, crystallization from aqueous ethanol gave (2S,3S)-2-acetamido-3-(2-amino-3-hydroxy-3H-indol-3-yl)propionic acid* in

* For the R,S designation, IUPAC nomenclature was used here instead of the conventional α,β designation for amino acids.

its hydrated form. The anhydrous material (66 mg, 12%, mp. 224-226°C (dec.)) was obtained from the hydrated material after drying in vacuo at 80°C overnight. $[\alpha]_D^{18°C}$ - 12.8° (c 0.5, 0.1M HCl). Anal. Calcd. for $C_{13}H_{15}N_3O_4$: C, 56.31; H, 5.45; N, 15.16. Found : C, 56.05; H, 5.42; N, 14.92. IR (KBr) : 3600-2200, 1720-1500, 1195, and 1143 cm^{-1}. UV (λ_{max}/nm (ε)) : pH<5, 264 (4140) and 297 (2850) ; pH>10, 272 (7500), 282 (5870), and 310 (2710). ^{1}H-NMR (DMSO-d_6, with a few drops of 1M ^{2}HCl) : 1.81 (s, 3H, $COCH_3$), 2.34-2.81 (ABX spin system (J=2.9, 9.9, and 13.9 Hz), 2H, β-CH_2), 3.79 (d.d., J=2.9 and 9.9 Hz, 1H, α-CH), 7.09-7.54 (m, 4H, arom.H), 8.20 (d, J=13.9 Hz, trace, unexchanged amide proton), and 10.15 (unexchanged COOH proton).

[8] N^{α}-Acetyl-β-(2-amino-3-hydroxy-3H-indol-3-yl)alanine amide (Ac-AHT-NH_2)

Ac-NFK-NH_2 (554 mg, 2 mmol) was dissolved in methanol (3 ml) with gentle warming. Solid potassium cyanide (130 mg, 2 mmol) and glycine (150 mg, 2 mmol) were added to this solution. The suspension was vigorously stirred for three hours until it became clear. Concentration of the solution gave the sirupy material, which was crystallized by the addition of a small amount of cold water. Colourless fine crystals of Ac-AHT-NH_2, mp. 187-190°C (dec.) were obtained in the yield of 220 mg (40%). Anal. Calcd. for $C_{13}H_{16}N_4O_3$: C, 56.51; H, 5.86; N, 20.28. Found : C, 56.20; H, 5.86; N,20.28. IR (KBr) : 3540, 3430, 3390, 1660, 1573, and 1075 cm^{-1}. ^{1}H-NMR

(DMSO-d_6) : 1.52 (s, 3H, $COCH_3$), 2.15 (d, J = 6.6 Hz, 2H, β-CH_2), 3.98 (q. or d.d., J = 6.6 and 7.6 Hz, 1H, α-CH), 5.96 (s, broad, 1H, OH), 6.67-7.20 (m, 6H, arom. H and amidine NH_2), 6.84 (s, 2H, $CONH_2$), and 7.31 (d, J = 7.6 Hz, 1H, α-NH). UV(λ_{max}/nm (ε)) : pH < 5, 264 (3760), 297 (2690); pH > 10, 273 (6530), 283 (5030), and all (2580). pKa (in ^2H_2O, ^{13}C-NMR titration of Ac-[2-^{13}C]ATH-NH_2) : 7.11 (data is shown in Fig. 2-13 of Chapter 2)

[9] N-[2-(2-amino-3-hydroxy-5-methoxy-3H-indol-3-yl)ethyl]-acetamide (AHM) from melatonin

At first, melatonin (232 mg, 1 mmol) was converted to N-[2-(2-Formamido-5-methoxybenzoyl)ethyl]acetamide by methanolic ozone oxidation. The yield was 140 mg (53%). Recrystallization from methanol-ether gave pale yellow needles, mp. 145-146°C. Mass : m/e = 264 (M^+).

N-[2-(2-Formamido-5-methoxybenzoyl)ethyl]acetamide (132 mg, 0.5 mmol) was dissolved in methanol (2 ml) and solid potassium cyanide (33 mg, 0.5 mmol) and glycine (38 mg, 0.5 mmol) were added to the solution. The mixture was stirred vigorously for several hours until the suspension became clear. The stirring was continued for additional one hour at room temperature. The clear solution was stored in a refregerator at -20°C for several days. Pale yellow thin plates separated out were filtered, washed with a small amount of methanol and dried in vacuo at room temperature. The yield of AHM was 121 mg (82%). The crystals thus obtained contained

one molecule of methanol of crystallization. Analytical sample was mp. 120-121°C (dec., evolution of methanol : > 110°C). Anal. Calcd. for $C_{13}H_{17}N_2O_3 \cdot CH_3OH$: C, 56.94; H, 7.17; N, 14.23. Found : C, 56.68; H, 7.07; N, 14.05%. MS (m/e) : 263 (M^+, 70%), 204 ($M^+ - CH_3CONH_2$, 100%). UV(λ_{max}^{MeOH} / nm(ε)) : 278 (12800) and 321 (2450). ^{1}H-NMR (DMSO-d_6) : 1.71 (s, 3H, acetyl CH_3), 1,83-2.14 (m, 2H, 2-CH_2), 2.64-2.97 (m, 2H, 1-CH_2), 3.68 (s, 3H, OCH_3), 5.78 (s, broad, 1H, 3-OH), 6.59-6.81 (m, 5H, arom.H and NH_2), 7.64 (d, J = 4.9 Hz, 1H, amide NH). Methyl and hydroxy proton signals of methanol were detected at 3.17 (3H) and 4.10 (broad 1H) ppm., respectively.

[10] Formation of benzyloxycarbonyl-L-alanyl-L-tryptophan (Z-Ala-Trp-OH) from Z-alanine o-acetophenylamide and tryptophan by transacylation.

Z-L-alanine o-acetophenylamide was prepared from Z-L-alanine and o-aminoacetophenone with DCC in tetrahydrofuran and recrystallized from methanol and ether (Yield: 75%, mp. 88°C). Anal. Calcd. for $C_{19}H_{20}N_2O_4$: C, 67.04; H, 5.92; N, 8.23. Found : C, 66.91; H, 5.84; N, 8.22. ^{1}H-NMR-(DMSO-d_6) : 1.36 (d(J = 6.59 H_z), 3H, Ala-β-CH_3), 2.65 (s, 3H, -CO$\underline{CH_3}$), 4.13 (d.q., 1H, Ala-α-CH), 5.09 (s, 2H, benzyl CH_2), 7.2-8.6 (m, 10H, arom.H + Ala-αNH), and 11.9 (s, 1H, acetophenyl $\underline{NH}$).

Z-L-Alanine o-acetophenylamide (680 mg, 2 mmol), L-tryptophan (424 mg, 2 mmol), and potassium cyanide (140 mg, 2.2 mmol) were dissolved in aqueous 80% (v/v) N,N-dimethyl-

formamide (12 ml). The solution was stirred for 2 days at 15-20°C. After evaporation of the solvent under reduced pressure, the residue was taken up in water (40 ml, turbid) and extracted with ethyl acetate (50 ml x 3). From the organic layer, the unreacted starting material (220 mg, 32%) was recovered. The aqueous layer was acidified to pH 2 and extracted again with ethyl acetate (50 ml x 3). The organic layer was washed with saturated NaCl solution (50 ml x 2), dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The residue was crystallized out by the addition of n-hexane. Recrystallization from aqueous ethanol gave Z-Ala-Trp-OH (269 mg, mp. 171-172°C) in 33% yield. Anal. Calcd. for $C_{22}H_{23}N_3O_5$: C, 64.53; H, 5.66; N, 10.26. Found: C, 64.42; H, 5.55; N, 10.10. ^{1}H-NMR (DMSO-d_6) : 1.19 (d(J = 7.32 Hz), 3H, Ala-β-CH_3), 3.0-3.3 (m, 2H, Trp-β-CH_2), 4.1 (m, d.q. (J = 7.32 Hz), 1H, Ala-α-CH), 4.5 (m, 1H, Trp-α-CH), 5.01 (s, 2H, benzyl CH_2), 6.9-8.0 (m, 10-11H, arom.H and amide NH), 10.8 (s, 1H, indole NH), and 12.6 (s, 1H, Trp-COOH).

[11] ^{13}C-Labeling of the indolic C-2 atom of N^{α}-acetyl-L-tryptophan in one pot.

N^{α}-Acetyl-L-tryptophan (492 mg, 2 mmol) was oxidized to N^{α}-acetyl-N'-formyl-L-kynurenine (Ac-NFK-OH) as described in p. 12. The sirupy potassium salt of Ac-NFK-OH and potassium cyanide (140 mg, 2.2 mmol) were dissolved in water (3ml). The solution was stirred overnight at room temperature.

After the formation of the AHT derivative was confirmed by its characteristic UV spectrum, 1M HCl (2.2ml) was added to the reaction mixture. The solution was then concentrated to dryness. After the residual mass was suspended in water (5 ml), sodium borohydride (400 mg, 11 mmol) was added to the suspension in several portions for 2 hours. The pH was kept below 10 with the occasional additions of acetic acid. The resulting clear solution was allowed to stand for 30 min, acidified with acetic acid to pH 4, and concentrated to about 1 ml. When the residue was taken up in cold 0.1M HCl (20 ml), almost colorless fine leaflets separated out. The yield was 291 mg (59%). Recrystallization from aqueous ethanol gave pure Ac-Trp-OH, mp. 186.5-187°C (lit (4) 189°C). Anal. Calcd. for $C_{13}H_{14}N_2O_3$: C, 63.40; H, 5.73; N, 11.38%. Found : C, 63.60; H, 5.83; N, 11.60%. $[\alpha]_D^{23°C}$ + 27.2° (c = 1, 1N NaOH) (cf. $[\alpha]_D^{23°C}$ + 28.0° for the starting material under the same conditions).

N^{α}-Acetyl-L-[2-^{13}C]tryptophan prepared according to this method in a smaller scale experiment gave the molecular ion peak (M^+) at m/e = 247 by EI-mass spectrometry and the single signal due to the enriched ^{13}C nucleus at 122.8 ppm (in DMSO-d_6) on the ^{13}C-NMR spectrum.

[12] Preparation of N-[2-([2-^{13}C]-5-methoxyindol-3-yl)ethyl]-acetamide ([indole-2-^{13}C]melatonin)

The ^{13}C atom was incorporated into N-[2-([2-^{13}C]-2-amino-3-hydroxy-5-methoxy-3H-indole-3-yl)ethyl]acetamide ([2-^{13}C] AHM)

from $K^{13}CN$ as described in [9].

[2-^{13}C]AHM (76 mg, 0.25 mmol) was dissolved in water (2 ml). To this solution was added solid sodium borohydride (100 mg, 2.6 mmol) in four portions with occasional additions of acetic acid to keep the pH of the solution below 10 during the reduction performed at room temperature. After the completion of the reaction was confirmed by the UV spectrum which showed the recovery of the characteristic 5-methoxyindole chromophore in an acidic solution, 1M HCl was added to the solution until the pH became below 3. The resulting slightly turbid solution was saturated with NaCl and extracted with ethyl acetate three times (5 ml, each). The organic layer was combined and concentrated under reduced pressure. The sirupy residue was crystallized by the addition of benzene (3 ml). The yield was 43 mg (74%), mp. 113-115°C (lit, (12) 115-116°C). Fig. 1-8 compares the EI-mass spectra of [2-^{13}C]melatonin thus obtained with the starting authentic (natural) melatonin. ^{13}C-NMR ($CDCl_3$) : 122.64 ppm (single resonance).

[13] Effect of amines on the formation of 2-amino-3H-indol-3-ols

The reaction of an N-acylanthraniloyl compound to cyanide anion was followed by measuring the absorbance at 281.5nm with various amounts of amines. A typical experiment is as follows.

The reaction mixture (3 ml) was made up of 3-(N-formylanthraniloyl)propionic acid (2 mM), potassium cyanide (40

mM), and glycine (0-0.5 M) in sodium phosphate buffer (0.2 M, pH 8.0). The reaction was carried out at 0-4°C. Aliquots (0.1 ml) were taken from the reaction mixture at appropriate intervals, diluted with water (3 ml) and recorded UV spectra. Similar experiments were carried out using methylamine, L-histidine, or L-proline in place of glycine.

[14] Reduction of 2-amino-3H-indol-3-ols with hydride reagents in dilute aqueous media under various conditions

In a series of experiments, Ac-(2S,3R)-AHT-OH was used as a model compound. The reaction medium (2 ml) was composed of Ac-AHT-OH (0.41 mg, 1.5 μmol) and some additives.

The freshly prepared ice-cold solution (0.05 ml) containing hydride reagents (80 μmol) and 3 M LiCl. The reaction was carried out at room temperature (20-25°C). Aliquots (0.2 ml) were withdrawn from the reaction mixture at time intervals and diluted with deionized water (3 ml) for UV measurements. The spectra were recorded before and after acidification with 1 M HCl (0.1 ml). The reaction yield was estimated from the molar absorption coefficient of Ac-Trp-OH at 280 nm (ε = 5600 $M^{-1}cm^{-1}$). When the yield was low (40-60%), the ratio of absorbance at 254 nm (A_{254}), an isosbesitic point for the reduction intermediate and the final product, to that at 280 nm (A_{280}) was taken. That is, the yield (Y) is calculated by the formula,

$$Y = \frac{A_{280} / A_{250}}{2.7} \times 100$$

where 2.7 is the value of A_{280} / A_{254} for authentic Ac-Trp-OH.

[15] Identification of the reduction intermediates of 3-(2-amino-3-hydroxy-3H-indol-3-yl)propionic acid

The reduction intermediates of 3-(2-amino-3-hydroxy-3H-indol-3-yl)propionic acid with sodium cyanoborohydride ($NaBH_3CN$) were analyzed by ^{13}C-NMR spectroscopy. The measurements were performed essentially in the same manner as described in the previous experiments with sodium borohydride (3). In the present experiment, the reduction was conducted with $NaBH_3CN$ in 3M LiCl-^2H_2O solution. Concentrations of 3-([2-^{13}C]-2-amino-3-hydroxy-3H-indol-3-yl)propionic acid and $NaBH_3CN$ were 12 mM and 50 mM, respectively. The unlabeled reduction intermediate (ca. 20 mg) was prepared by essentially the same manner as above. Samples for ^{13}C-NMR measurements were prepared as follows. The reduction intermediate was first extracted with ethyl acetate from the reaction mixture and then re-extracted into dilute alkaline solution containing an equimolar amount of NaOH to the intermediate. After neutralization with 1M HCl, the solution was lyophilized. The residual mass was dissolved in ^2H_2O and directly submitted to the ^{13}C-NMR measurement.

RESULTS

[1] Preparation of N-acylanthraniloyl compounds and their reactions with cyanide.

To obtain N-acylanthraniloyl compounds, there are mainly two pathways : ozone oxidation of indoles and acylation of anthraniloyl compounds. The former is suitable for the present labeling method because the starting material and the final indole compound labeled are usually the same when the indole has no functional group(s) reactive to reagents used in the labeling reactions. The latter method provides a new route for the indolization of anthraniloyl compounds. In this study, the former method established by Masuda et al (8) was mainly used because it is simple, nearly quantitative and easy to work up. However, the N-acyl derivatives of anthraniloyl compounds other than the N-formyl ones were prepared by the latter acylation method.

Reactions of several anthraniloyl compounds to potassium cyanide were examined under various conditions. Results of these experiments are summerized in Table 1-1.

(Table 1-1)

This Table clearly shows the anomalous reactivity of the N-formyl derivative to the cyanide. The acetyl derivative reacted with cyanide only in the presence of a primary amine,

which was acetylated during the reaction. In non-aqueous media, the presence of a neutral alkylammonium salt was essential for this reaction. When free alkylamine was used, many by-products were formed. Methylamine hydrochloride and amino acids, e.g., glycine, were effective as additives.

The rate of reaction in aqueous media was dependent on concentrations of reactants including these additives, pH, and temperature. The effects of pH and the addition of glycine on the reaction rate are shown in Fig. 1-1.

(Fig. 1-1)

This Figure shows that the addition of glycine accelerates the reaction rate significantly at neutral pH's. The time-course of the UV spectral change is also shown in Fig. 1-2.

(Fig. 1-2)

The undesirable hydrolysis of the reaction product, 2-amino-3H-indol-3-ol, to the corresponding 2,3-dioxindole derivative was suppressed almost completely when the reaction was carried out at 0-4°C.

Two other reactions were found in the present study. One is the formation of 2-iminopyrrolidin-3-ol derivatives from N-[2-(3-aminopropionyl)phenyl]formamide (N'-formylkynurenamine) and N'-formylkynurenine (13).

The other is the formation of cyclopenta[b]quinolin-

4-one from 1-aza-8,9-benzocyclononene-2,7-dione derived from 1,2,3,4-tetrahydrocarbazole. This reaction occurred also in basic media without KCN. (14).

[2] Structure and reactions of 2-amino-3H-indol-3-ols

The structure of 2-amino-3H-indol-3-ol was analyzed by the X-ray crystallography of one of diastereoisomers of Ac-AHT-OH by Dr. Y. Hata et al. of Institute for Protein Research, Osaka University. Fig. 1-3 shows the stereoview and several molecular parameters of this compound.

(Fig. 1-3)

It was demonstrated that 2-amino-3H-indol-3-ol was reactive to carbonyl reagents (15 , 16), hydride reagents, and hydroxide anion (13). These reactions resulted in a substitution of the 2-"imino" group. However, the 2-amino-3H-indol-3-ol did not always behave as a typical carbonyl compound, as shown by the fact that it reacted neither with the excess cyanide anion nor with benzaldehyde or p-dimethylaminobenzaldehyde in the presence of cyanide (benzoin condensation, data not shown). On the other hand, the formal 2-"amino" group was highly resistant to acylation with acid anhydrides or with activated esters and to alkylation with α-haloacetic acids in aqueous solutions.

These results suggest that the properties of 2-amino-3H-indol-3-ol are represented by the amidine structure around the C-2 position.

[3] Detection of intermediates of 3-(2-amino-3-hydroxy-3H-indol-3-yl)propionic acid with sodium cyanoborohydride

When the ^{13}C-NMR spectrum of the reaction mixture of 3-([2-^{13}C]-2-amino-3-hydroxy-3H-indol-3-yl)propionic acid and $NaBH_3CN$ was measured, three signals* corresponding to the C-2 carbons of intermediates and the final indole compound were detected at 181.7, 123.3, and 58.9ppm, as shown in Fig. 1-4.

(Fig. 1-4)

From the chemical shift, the signal at 123.3ppm was readily assigned to 3-(indol-3-yl)propionic acid. The signal at 58.9ppm was due to the methylene carbon of 3-hydroxyindoline derivative as reported elsewhere (3). The signal at 182ppm was detected first in this experiment and was shown to split into a doublet under the off-resonance proton decoupling condition (data not shown), indicating that the carbon in question bears one proton. This signal was also detected on the natural abundance spectrum of the almost pure intermediate.

[4] Optimal conditions for the hydride reduction of 2-amino-3H-indol-3-ols in highly dilute aqueous solutions

To reduce 2-amino-3H-indol-3-ol in peptides and proteins with hydride reagents, it is necessary to carry out the reaction in highly diluted aqueous solutions. In model reactions, N^{α}-

* These signals were formed accidentally but conveniently because of the excessive proton-irradiation of the highly polar (3M LiCl) solution in the NMR probe caused it to be overheated.

acetyl-(2S,3R)-3-(2-amino-3-hydroxy-3H-indol-3-yl)alanine was reduced in 1-10mM solutions with 1 to 80-fold molar excess of hydride reagents, i.e., $NaBH_4$, $LiBH_4$, and $NaBH_3CN$. In these dilute solutions, decomposition of $NaBH_4$ (or $LiBH_4$) exceeded the desired reduction of the 3H-indole, probably because the decomposition rate was pseude-first order in the hydride concentration whereas the reduction rate is second or third order. However, the use of high concentrations of a reducing agent should be avoided to reduce 2-amino-3-indol-3-ol in a protein. Therefore, several compounds were added to reaction media to enhance the reaction rate and yield. Table 1-2 summerizes the effect of the additives on reaction parameters.

(Table 1-2)

Fig. 1-5 and Fig. 1-6 show the effects of LiCl and ethylenediaminetetraacetic acid (EDTA) on reaction yields and rates

(Fig. 1-5)

(Fig. 1-6)

As shown by these figures, LiCl and EDTA (neutral or acidic salt) were found to be effective for the reduction and to contribute the reaction in a different manner. LiCl had an enhancing effect on the reaction yield. The similar effect was found for NaCl and guanidium chloride. However, KCl and other halides, acetate, sulfate of metal ions, had no or negative effects. Another useful additive, EDTA, increased the reaction

rate significantly. EDTA could not be replaceable with ethyleneglycol, ethylenediamine diacetate, ethyleneglycol monomethyl ether, and 2-mercaptoethanol. The effects of LiCl (or NaCl, guanidium chloride) and EDTA became maximal at 3-4 M and 0.2%, respectively. Thus, the combine use of LiCl and EDTA in these concentration ranges was satisfactory. The combination of EDTA and the chloride cited above was also effective in the reduction with $LiBH_4$, with which the reduction of 2-amino-3H-indol-3-ol was only 5% less effective compared with $NaBH_4$ in the presence of these compounds. The reduction was unsuccessful without EDTA even in the presence of the chloride.

The "normal" reduction intermediate with λ_{max} 235 and 290nm observed under the above conditions was not detected by UV spectroscopy upon reduction with $NaBH_3CN$. Instead, another reduction intermediate with λ_{max} 262 and 296nm was found. This "abnormal" intermediate was further converted to the "normal" one upon addition of $NaBH_4$. The "normal" intermediate only could afford the final indole compound by acidification. The total yield was comparable to that of the reduction with $NaBH_4$ in the presence of LiCl and EDTA. Other factors to affect the reduction with $NaBH_4$ are pH and temperature. Especially the influence of pH is significant. The medium in which the reaction attained the highest yield and rate contained 3M LiCl and 0.2% EDTA·2Na. In this medium, the reaction mixture was initially acidic (pH≈4) while it became basic (pH≈8) in the end. However, when the pH was fixed at a value between 6.5 and 8,

reaction yields were generally low. Unexpectedly, the yield was higher in acidic media than in basic ones. Even under these constant pH conditions, the yield was about 20% higher in the presence of EDTA than in the absence.

The effect of temperature was less significant than that of pH. The yields of Ac-Trp-OH were 80% and 67% at 37°C and 0°C, respectively, in the reduction of Ac-AHT-OH in the presence of 3M LiCl and 0.2% EDTA·2Na.

[5] Preparation of [2-^{13}C]indole derivatives

N^{α}-Acetyl-L-tryptophan and melatonin (N-[2-(5-methoxyindol-3-yl)ethyl]acetamide) were labeled with ^{13}C at their indole C-2 positions under the conditions established in the present study. Especially, N^{α}-acetyl-L-[2-^{13}C]tryptophan could be prepared essentially in one pot without racemization. Melatonin was the first 5-substituted derivative of indoles to which the labeling reaction was successfully applied.

The ^{13}C-NMR spectra of N^{α}-acetyl-L-tryptophan with and without ^{13}C-labeling are shown in Fig. 1-7.

(Fig. 1-7)

The enriched ^{13}C signal appeared at 122.8ppm. The mass spectra of melatonin, with and without ^{13}C-enrichment, are shown in Fig. 1-8.

(Fig. 1-8)

DISCUSSION

[1] Reaction mechanism for the cyclization of N-acylanthraniloyl compounds to 2-amino-3H-indol-3-ols with cyanide.

Early kinetic study has indicated that the rate of this cyclization did not obey the simple second-order kinetics (16). In fact, the previous observation that the reaction proceeded only in aqueous media has implied the participation of the third factor, a nucleophile accepting the formyl group. This idea was verified by the present experiments in non-aqueous media containing an alkylammonium salt in addition to two reactants, N-formylanthraniloyl compounds and the cyanide. The reaction products were the expected 3H-indole derivative and the formylated amine. Furthermore, it was shown that the reaction took place for N-acetyl derivative of anthraniloyl compound in the presence of an acyl-accepting substance in both aqueous and non-aqueous media.

In this cyclization, any stable reaction intermediates were not detected so far. Furthermore, all attempts to prepare the suspected reaction intermediate, 1-acyl-3-alkyl-2-iminoindolin-3-ol by acylating the corresponding 3H-indole derivative were unsuccessful. This may be due either to the resistance of the 2-amino-3H-indole to the N-acylation or to the instability of its N-acylated derivative, or both. Evidently, these properties are responsible for the peculier ionic character of the amidine moiety with the pKa values of 6.5-7.5. It is known that compounds having neutral pKa values can act as good leaving groups in the reaction of their acylated derivatives

with nucleophiles, as shown by activated esters and N-acyl-imidazoles (18). Table 1-3 lists pKa values of these typical leaving groups.

(Table 1-3)

The reactivity of N-acylanthraniloyl compounds to cyanide depends on the character of the N-acyl substituents. Table 1-1 shows that the reaction is most favourable with the formylated derivative and that the acylated one follows. No reaction occurred for unacylated compounds. These results support the idea proposed by Bell and Wei (15), who has claimed that the driving force for the cyclization reaction is mainly ascribed to the electron-withdrawing effect of the acyl substituent, based on the results of experiments using acylaminobenzophenones including the dichloroacetyl derivatives. In their case, no reaction occurred without acylating the aromatic amino group. However, Bell and Wei did not realize the significance of a nucleophile as an acceptor of the acyl group. Therefore, it is reasonable that the reaction of N-acetylaminobenzophenone with cyanide is unsuccessful under their experimental conditions. The reaction might have occurred if an appropriate nucleophile were added to the reaction medium.

According to the present study, it is highly suspicious that electron-withdrawing property of N-acyl substituents is the most important factor in the 3H-indole formation. This view cannot explain the fact that o-(trifluoroacetylamino)acetophenone is

much less reactive to cyanide than the formamide derivative.

Consequently, it is concluded that the deacylation step is rate-determining in this reaction and that the acyl group may serve to stabilize the anionic state of the acylamino nitrogen atom which subsequently attacks the nitrile carbon in the cyanohydrin moiety formed by the addition of the cyanide anion to the carbonyl function. The deprotonation of amide nitrogen to bear a negative charge may be induced by the cyanohydrin formation in which the carbonyl oxygen abstracts the amide proton. The stabilization of the anion thus formed by the acyl substituent may be accomplished by delocalizing the negative charge. The innertness of the trifluoroacetyl derivative to cyanide is interpreted by the idea that the trifluoroacetyl group reduces the nucleophilicity of the induced anionic nitrogen by its own strong electron-withdrawing nature. Fig. 1-9 shows a scheme for the mechanism of the reaction deduced from the present investigation.

(Fig. 1-9)

This scheme involves charged intermediates, which may be favourably formed in polar media than in non-polar organic solvents. This assumption agrees with the fact that the reaction proceeds much faster in aqueous media than in methanol. Unfortunately, the reaction in other organic solvents was limited because of the low solubilities of potassium cyanide and primary alkylammonium salts in those less polar media.

The scheme also suggests another interesting role of an ammonium salt. When the anionic amide nitrogen of the intermediate (II) attacks the nitrile carbon, the resulting anionic intermediate (III) should require a proton donor for the neutralization of a negative charge. It is feasible to infer that the removal of an amine proton from the alkylamine results in the activation of the amino nitrogen for attacking the N-acyl group of (III) simultaneously. Thus, it is reasonable that any one of these intermediates has not been detected.

What will happen if N-acylanthraniloyl compound has a primary amino group? When 2-(N-formylanthraniloyl)ethylammonium chloride was subjected to the reaction with potassium cyanide either in water or in an organic solvent, the cyclization took place in a different manner and yielded 2-iminopyrrolidin-3-ol. This reaction had been thought to proceed *via* the electrically neutral cyanohydrin intermediate. However, if the formation of anionic intermediate (III) in the cyclization is assumed (Fig. 1-9), it is likely that this is a key intermediate which cyclizes either to 2-iminopyrrolidin-3-ol or to 2-amino-3*H*-indol-3-ol. The revised mechanism is shown in Fig. 1-10. This mechanism clearly explains the anomalous reactivity of the intramolecular primary amino group in the cyclization reaction.

(Fig. 1-10)

[2] Application of the 3H-indolization reaction to the coupling of a carboxylic acid and a primary amine

According to the reaction mechanism proposed in Fig. 1-9, the 3H-indole formation accompanies the activation of both the acyl portion of N-acylanthraniloyl compound and an added amine. Therefore, it is reasonable to suppose that this reaction is applicable to the formation of peptide bonds. The principle of this coupling reaction is shown in Fig. 1-11.

(Fig. 1-11)

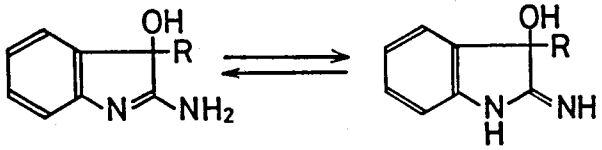
Actually the reaction was demonstrated by the preparation of Z-Ala-Trp-OH. The advantage of this amide bond formation, or transacylation, is that the reacting species are activated in situ in a concerted manner, and that the requirement of the reaction for both acyl and amine components considerably reduces the opportunity of side reactions. Conversely, no reaction can occur without one of these components and cyanide. These advantages are very attractive especially for the coupling of large peptide fragments. In this reaction, the amino group of one fragment and cyanide must attain to the N-(o-acetophenyl)amidated carboxyl terminus of another peptide fragment at the same time, otherwise these species remain unchanged.

Thus, this new coupling method utilizing the stability and highly restricted reactivity of o-acetophenylamido group appears superior to the conventional DCC (19), activated ester (20), and azide (21) methods for peptide synthesis.

In practice, however, several improvements are necessary for the general use as a method of peptide synthesis. First, the coupling reaction is very slow in non-aqueous media owing to low solubilities of potassium cyanide and the amine component which should be added as an ammonium salt. In contrast, in aqueous media, low solubility of the acyl component may also surpress the reaction rate. To overcome solubility problem, the introduction of a polar functional group into the o-acetophenylamide moiety may be necessary for reactions in aqueous media. If all the reactants become soluble in water, this method may be applicable to semi-synthesis of large polypeptides (and therefore proteins) in aqueous media. Second, it is expected that the introduction of a moderately electron-withdrawing group at para-position to the acylamino group may serve to increase the reaction rate. Attempts to introduce an appropriate functional group for these purposes are now in progress. Additionally the racemization of the acyl component is to be studied when this method is used actually.

[3] Structure and properties of 2-amino-3H-indol-3-ols

Theoretically the amidine moiety of 2-amino-3H-indol-3-ols is written as two tautomeric forms, 2-aminoindolenine (A) and 2-iminoindoline (B).

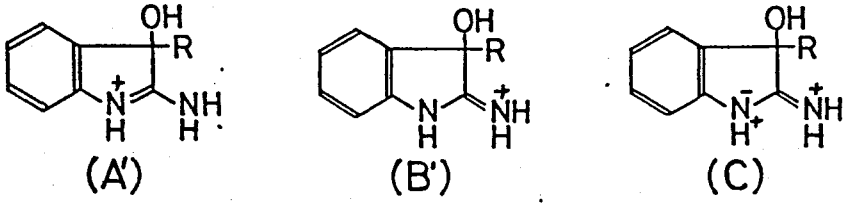


(A) (B)

From chemical and spectroscopic evidence, the predominance of the imino form (B) in the electrically neutral species is suggested. The amidine moiety is reactive to the nucleophiles at the C-2 carbon and undergoes the substitution of the 2-"amino" group, as shown in Fig. 1-12

(Fig. 1-12)

For the protonated form, the X-ray crystallographic analysis of N^{α}-acetyl-(2S,3R)-3-(2-amino-3-hydroxy-3H-indol-3-yl)alanine gave the following interesting result. The analysis revealed that the protonated imino (B') structure is predominant because the Cl*-N3* bond length (0.129 nm) is shorter than the Cl-Nl* distance (0.134 nm)(see Fig. 1-3). This finding suggests that the zwitter-ion structure should have a proton (not observable) at the Nl atom because the other two hydrogen atoms are present on the N3 nitrogen. These are contradictory results with respect to the positions of positive charge and of double bond in the amidine moiety. However, it is conceivable that the positive charge is mainly distributed between the N3 nitrogen and the N-1 hydrogen atoms. Hence, the protonated (B') form should be described as a resonance hybrid represented by the following canonical structures.



*According to arbitrary numbering shown in Fig. 1-3, Cl, Nl, and N3 correspond to the C-2 carbon, N-1 nitrogen and C-2 nitrogen atoms, respectively.

The canonical structure (C) is meaningful since it explains not only the X-ray crystallographic data but also chemical and spectroscopic properties of 2-amino-3H-indol-3-ols. For example, if structure (C) dominates over others, the C-2 carbon may be positively charged only weakly. In ^{13}C-NMR spectra, the protonation of the amidino group results in the upfield shift of the C-2 carbon resonance, contrary to the behavior of other cationic carbons in imidazolium and guanidium carbons. The increased reactivity of the C-2 carbon to necleophiles in the electrically neutral species relative to the cationic ones supports this idea. Additionally, the fact that the reaction of 2-amino-3-methyl-3H-indol-3-ol with acetic anhydride in acidic media yielded 1-acetyl-3-methyl-2,3-dioxindole* demonstrates the stronger nucleophilicity of the N-1 than the N-3matom as predicted from structure (C). The participation of this structure in the reduction with hydride reagents will be discussed in the next section.

It is important to realize, therefore, that 2-amino-3H-indol-3-ols display their properties and reactivities based on their different ionic states, i.e., cationic and neutral states.

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* This compound had been erroneously identified as 2-acetoxy-3-methyl-3H-indol-3-ol in a previous report (16), however, it should be corrected to 1-acetyl-2,3-dioxindole because the IR absorption at 1773 and 1670 cm^{-1} showed the presence of imine structure.

[4] Reduction of 2-amino-3H-indol-3-ols with hydrides

In the reduction of 2-amino-3H-indol-3-ols, a derivative of 3-hydroxyindoline was identified by ^{13}C-NMR and UV spectroscopy, as reported previously (3). However, the route from 2-amino-3H-indole-3-ol to this intermediate has not been clarified completely. Nevertheless, the following consideration is possible concerning the mechanism of reduction. The first stage of the reaction is probably concerned with the ionic interaction of the borohydride anion with the cationic 2-amino-3H-indol-3-ol in structure (B') or (C), since the reaction favoured acidic conditions at the initial stage. In fact, no reaction took place above pH 11. The enhancement of reaction yields by some chlorides may partly be interpreted by the idea that they are capable of producing an appropriate ion atmosphere for stabilizing the dipolar structure (C). If this is the case, the borohydride anion will attack the exo-C-2-nitrogen (N3) and then the hydride (H^-) will shift onto the C-2 carbon atom yielding the first intermediate, 2-amino-3-hydroxyindoline (IV). Hence the intermediate (IV) is apparently a simple addition product whose formation follows the addition-elimination mechanism as predicted in the previous section. Fig. 1-13 illustrates a possible sequence of the reactions. The first reduction product, 2-amino-3-hydroxy-

(Fig. 1-13)

indoline (IV), then spontaneously deaminates to 3-hydroxy-3H-

indole (V).

In the reductions of 2-amino-3H-indol-3-ols with either sodium cyanoborohydride or borohydride, the path leading to (V) is probably same. However, the reducing power of sodium cyanoborohydride was not strong enough to reduce 3H-indole (V) further to 3-hydroxy-indoline (VI), while at high temperature over 60°C, reduction of a part of (V) to (VI) was observed when it was followed by ^{13}C-NMR spectroscopy. The higher reactivity of sodium borohydride than the cyano derivative is obvious from the fact that (V) generated by the reduction with sodium cyanoborohydride was instantly converted to (VI) by the addition of sodium borohydride. The structure of intermediate (V) was elucidated by UV and ^{13}C-NMR spectroscopy for the reduction product of 3-(2-amino-3-hydroxy-3H-indol-3-yl)propionic acid with sodium cyanoborohydride. The indole C-2 carbon resonance was detected at 182 ppm (a doublet under off-resonance decoupling condition). The assignment of this signal was achieved by comparing the spectrum with that of the 2-^{13}C-enriched material. The UV spectrum (λ_{max} = 264 and 296 nm, pH-independent) of (V) demonstrated the presence of an indolenic (3H-indole) chromophore resembling 2-amino-3H-indol-3-ol (λ_{max} 265-275 nm and 300-310 nm, pH dependent) or dioxindoles (λ_{max} 255 and 300 nm, pH-independent). The detection of (V) as an reduction intermediate suggests strongly that the reaction really proceeds according to the addition-elimination mechanism shown in Fig. 1-9.

Furtheremore, there exists the possibility that a successive use of sodium cyanoborohydride and sodium borohydride results in a partial isotope labeling of the C-2 hydrogen atom of indoles.

Finally, the problem remained to be resolved is how EDTA contributes to the enhancement of reaction rate. The chelating of EDTA to metal ions had been supposed to be the cause but the addition of an excess of metal ions such as Fe^{2+} and Cu^{2+} did alter neither reaction rates nor yields (data not shown). It is unlikely that EDTA traps borate ions, which are formed by the decomposition of the reducing agent, and are considered to obstruct the reaction by competing with the hydride to interact with the substrate molecule, must be denied becasue other reagents, eg., ethylenglycol, failed to exert a similar effect.

The modification of hydride reagents with EDTA was implicated by a rapid evolution of hydrogen regardless of the presence and absence of the substrate. The reaction rate was dependent on EDTA concentration. Ethylenediamine had an effect similar to EDTA, although not so remarkable. From this finding and the proposed mechanism for the $NaBH_4$-reduction of 2-amino-3H-indol-3-ol (Fig. 1-13), it is reasonable to suppose that a more active species of the borohydride anion is generated in the presence of EDTA.

Consequently, the roles of additives, the chlorides and EDTA, and proton, are summarized as follows. First, the

chloride of lithium, sodium, or guanidium at high concentrations may stabilize the cationic form of 2-amino-3H-indole by forming a certain ion atmosphere in the vicinity of the amidine moiety. Second, EDTA increases the reactivity of hydride reagents to 2-amino-3H-indoles. The carboxylate anions of EDTA may serve to restore a negative charge on the "modified" borohydride, which readily interacts with the cationic substrate. Highly polar atmosphere provided by the salt may be also favourable to such an ionic interaction.

Since these additives are necessary for the reduction of the substrate at mM concentrations, the present reaction to form tryptophan from AHT would not be applied to proteins if the efficient additives had not been found.

[5] Dehydration of 3-Hydroxyindoline to Indole

The reduction product, 3-hydroxyindoline, readily lost a water upon acidification and yielded the corresponding indole. This process requires a proton which may catalyze the elimination of water. The elimination is very fast and apparently independent of temperature. Although the cis-elimination (probably via a benzyl cation) of water for indolization has been known (22) , trans-elimination is also possible and likely to occur in this case. The problem, whether cis- or trans-elimination is predominant in the dehydration of 3-hydroxyindolin, will be solved if a stereoselectively ^{2}H-labeled intermediate (IV) is prepared by

reducing 2-amino-3H-indol-3-olwith $NaBH_3CN$ and then NaB^2H_4 or with NaB^2H_3CN and then $NaBH_4$, successively.

[6] Concluding remarks

The reaction pathway which starts from and ends at indoles involves two stable interemediate compounds and several unstable or unidentiifed intermediates. Table 1-4 summarizes

(Table 1-4)

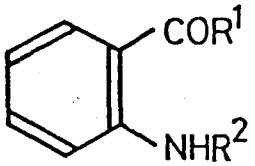
characteristic properties of these substances.

Since this pathway forms a reaction cycle, the interconversion of any compounds included in the cycle is possible. Consequently, the reaction scheme for the synthesis of an indole derivative utilizing this pathway may be designed. It is also possible to protect an indole nucleus from undesirable oxidation during a synthetic work by converting it temporarity to an inert compound involved in the reaction cycle.

Above all, this reaction cycle can be used for the isotope labeling of indole C-2 atoms. Especially the cycle is useful for the labelings of optically acitve or complex bioactive indole compounds, which are difficult to synthesize. The mildness and the relatively high yield of the reaction are characteristic of the present reaction. An example will be described in the next chapter, which deals with the ^{13}C-labeling of a tryptophan residue in hen egg-white lysozyme.

Table 1-1 Reactivities of various anthraniloyl compounds to potassium cyanide in various media.

Compound*1	Reaction Medium / Product			
	H_2O	H_2O+R'NH_2	MeOH	MeOH+R'NH_2
R^1=Alkyl R^2=H	—*2	——	——	——
R^1=Alkyl R^2=CHO	3H-Indole	3H-Indole	——	3H-Indole
R^1=Alkyl R^2=$COCH_3$	——	3H-Indole	——	3H-Indole
R^1=Ph *3 R^2=$COCHCl_2$	3H-Indole	No Data		
R^1=CH_3 R^2=$COCF_3$	——	——	——	——
R^1=OMe, OH R^2=CHO	——	——	——	——
R^1=$CH_2CH_2NH_2$ R^2=CHO	N-[2-(3-Hydroxy-2-iminopyrrol-3-yl)phenyl]formamide			
R^1}-CH_2CH_2 *4 R^2}-$COCH_2$	Cyclopenta[b]quinolin-4-one *5			

*1 

*2 No Reaction

*3 Ref. (15).

*4 1-Aza-8,9-benzcyclononane-2,7-dione

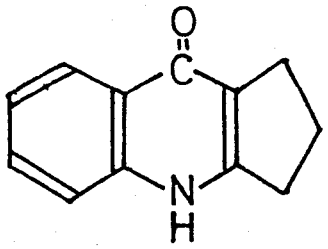
*5 

Table 1-2. Effects of additives on the reduction yields and rates of Ac-AHT-OH† with $NaBH_4$††.

Additive	Yield (%, maximal)	Rate (min*1)
none	20	> 60
3M LiCl (or NaCl, Gua·HCl)	50-60	> 120
3M KCl*2	30	> 120
3M NaF	-*3	
0.2% EDTA·2Na	60	< 5
0.05M Phosphate*4 (pH 7.5)	30	< 5
0.05M Phosphate (pH 7.5) + 0.2% EDTA	50	< 5
0.2% EDTA·2Na, + 3M LiCl	70-80	< 5
0.04% EDTA·2Na + 3M LiCl	70-80	> 60
0.2% en·2AcOH*5 + 3M LiCl	40	< 5
2.5% ethyleneglycol + 3M LiCl	65-75	> 180
2.5% ethyleneglycol + 0.2% EDTA·2Na + 3M LiCl	70	< 5
2.5% 2-mercaptoethanol + 0.2% EDTA·2Na + 3M LiCl	30	< 5

*1 Time consumed until the reaction attains 80% of the maximal yield.

*2 Na_2SO_4, LiOAc, KBr, NaI, and so on has similar effect.

*3 Reacted with the starting material (UV change).

*4 Sodium salt.

*5 Etylenediamine diacetate

† 0.75 mM

†† 30 mM

Table 1-3. pKa values of 2-amino-3H-indol-3-ol and the leaving groups of acylating agents.

Compound	pKa
2-Amino-3H-indol-3-ol	6.0-7.5
p-nitrophenol	7.15
imidazole	6.95

Table 1-4. UV absorption maxima and ^{13}C chemical shifts of the compounds related to the labeling reaction of indole C-2 atoms.

Compound	UV ($\lambda_{max}^{H_2O}$/nm)	^{13}C-NMR (C-2, ppm)
3-Alkylindole (Trp)	280	120-125
N'-Formylkynurenine (NFK)	260, 325	-
2-Amino-3H-indol-3-ol	265, 300	173-175 (H^+ form)
"	270, 310	175-178 (neutral)
3H-Indol-3-ol (V)	264, 296	182
3-Hydroxyindoline (VI)	235, 290	58-60
Kynurenine (Kyn)	260, 365	-
2,3-Dioxindole	255, 300	180-182

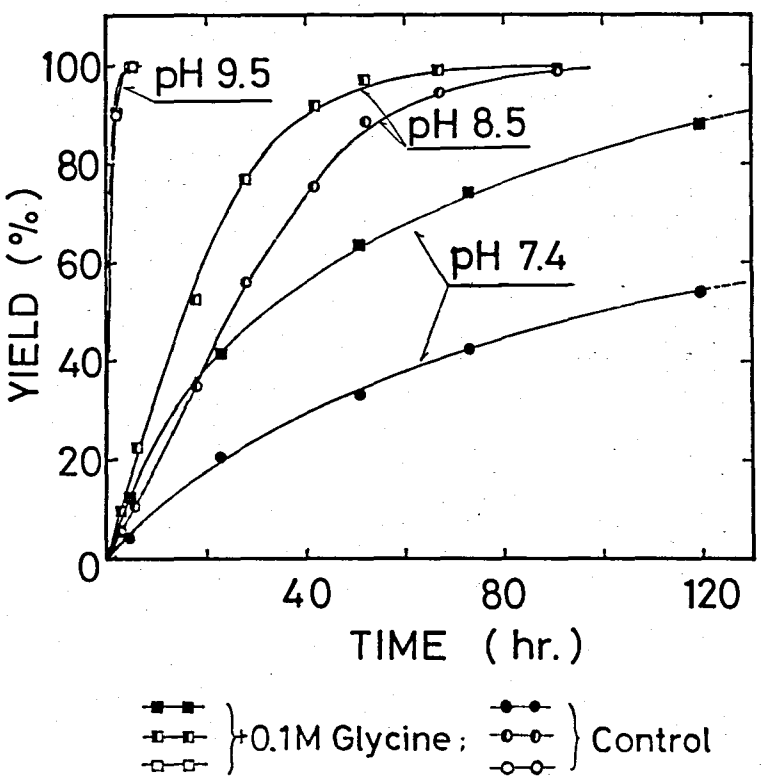


Fig. 1-1. The effects of pH and glycine on the reaction rate. The yield was calculated from the increase of absorbance at 281.5 nm (shoulder) based on the absorbance of the authentic 3-(2-amino-2-hydroxy-3H-indol-3-yl)propionic acid at the same concentration. Initial concentrations of 3-(N-formylanthraniloyl)propionic acid and potassium cyanide were 20 and 40 mM, respectively.

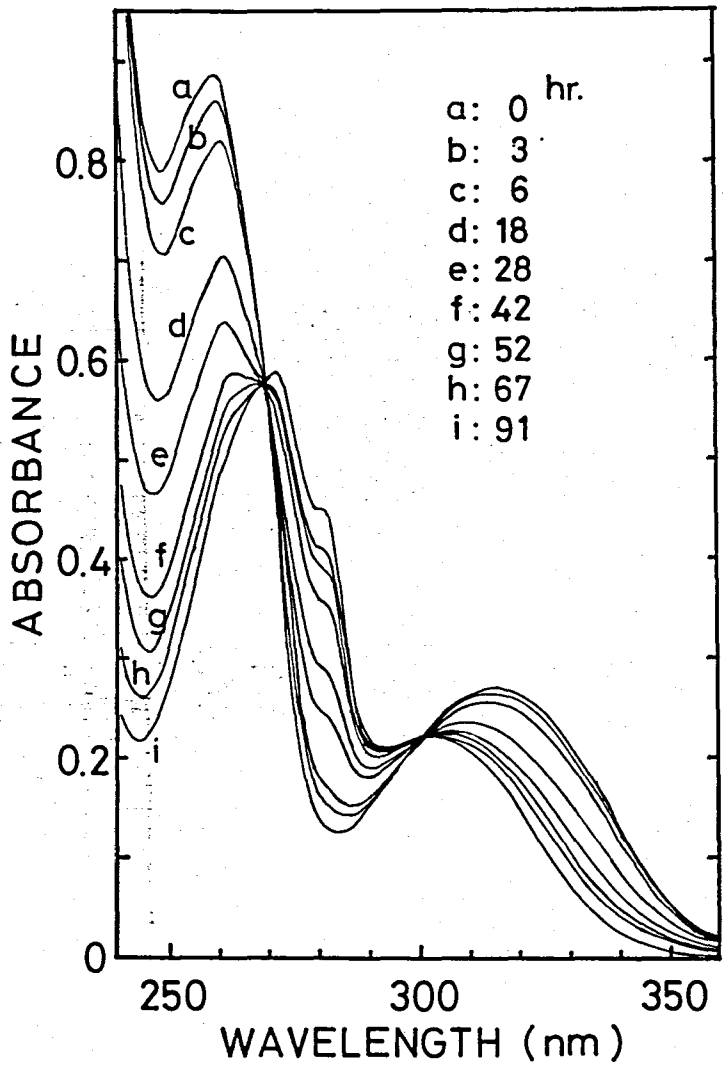


Fig. 1-2. The time-course of the UV spectral change during the reaction of 3-(N-formylanthraniloyl)propionic acid (2 mM) with potassium cyanide (40 mM) in the presence of glycine (0.05M) at pH 8.0 and 0°C.

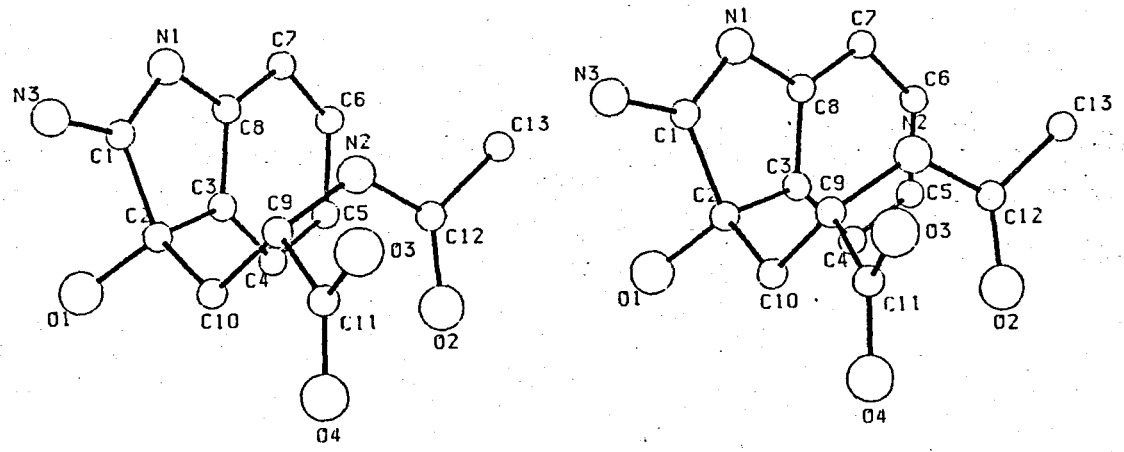


Fig. 1-3. Stereoview of N^{α}-acetyl-(2S,3R)-3-(2-amino-3-hydroxy-3H-indol-3-yl)propionic acid. Bond lengths and bond angles for amidino atoms are as follows. Positions of hydrogen atoms attached to N3 atom can be specified from these data.

Bond	Distance (nm)	Bond	Distance (nm)
N1 - C1	0.133	N3 - C1	0.129
N1 - C8	0.141	C1 - C2	0.155
N3 - H11	0.100	N3 - H12	0.099

Bond	Angle (°)	Bond	Angle (°)
N1 - C1 - N3	124.9	N1 - C1 - C2	110.2
N3 - C1 - C2	124.9	C8 - N1 - C1	110.6
C1 - N3 - H11	121.7	C1 - N3 - H12	124.3
H11 - N3 - H12	113.9		

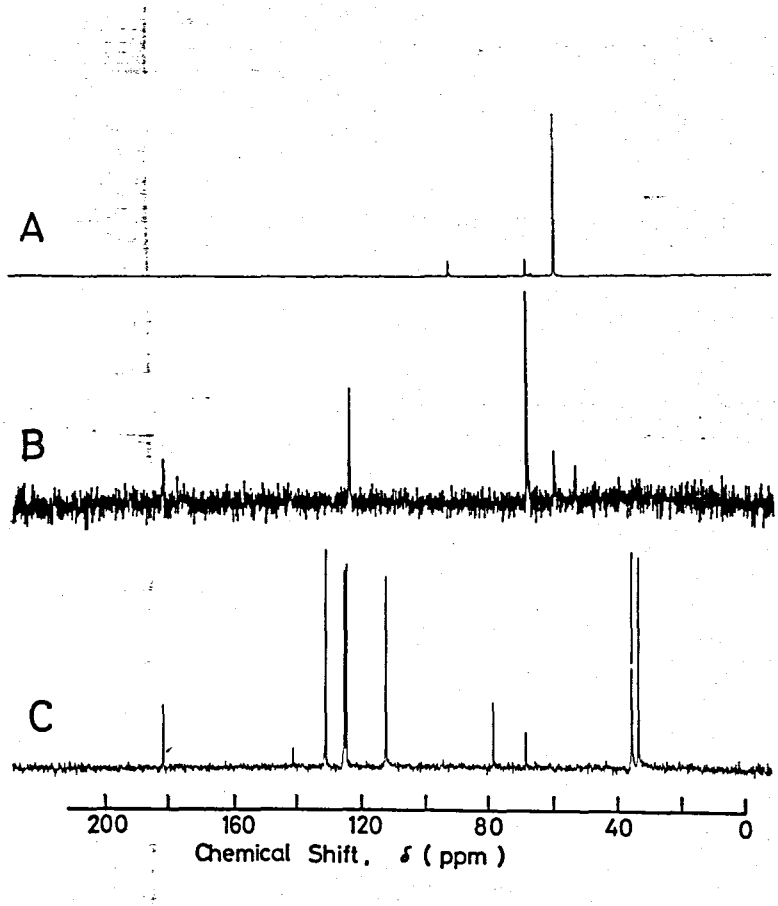


Fig. 1-4. ^{13}C-NMR spectra of reduction intermediates of 3-[2-^{13}C]-2-amino-3-hydroxy-3H-indol-3-yl)propionic acid with sodium borohydride (A) and with sodium cyanoborohydride (B). Spectrum (C) was recorded for the intermediate without ^{13}C-enrichment which was formed by the reduction with sodium cyanoborohydride. Spectra were accumulated 1000-2000 times with repetition of 1.5 s at 25.01 MHz. The signal at 67.4 ppm is internal dioxane in $^{2}H_2O$.

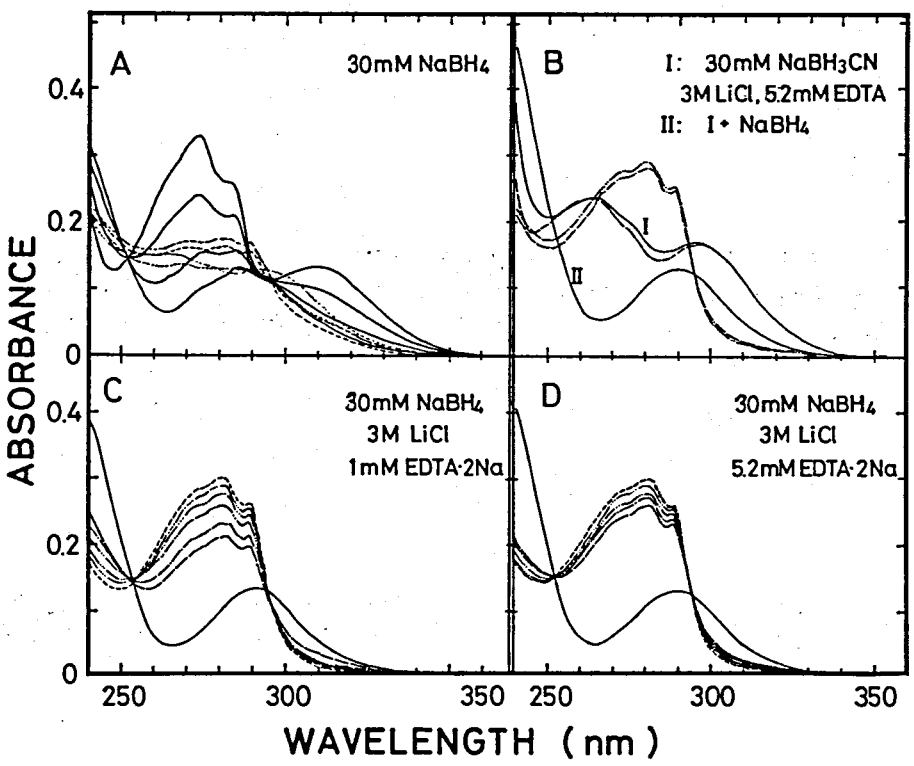


Fig. 1-5. UV spectral changes in the reduction of Ac-AHT-OH with hydride reagents to Ac-Trp-OH via the 3-hydroxyindoline intermediate (line II of B). Reaction intermediates are presented by solid lines and the product by broken lines which are recorded after acidification with 1 M HCl (0.1 ml). For (C) and (D), — — — — and ------ are recorded at 5 and 120 min., respectively. Reaction conditions, see "METHODS".

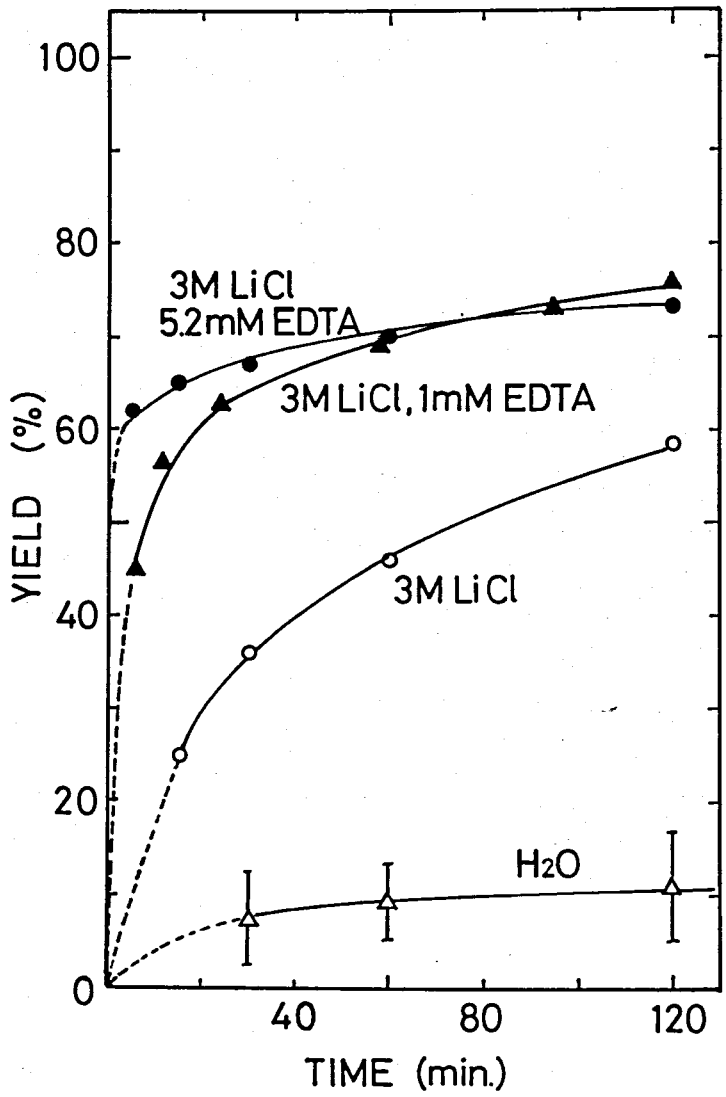


Fig. 1-6. Effects of LiCl and EDTA on reaction yields and rates. Reaction conditions, see "METHODS".

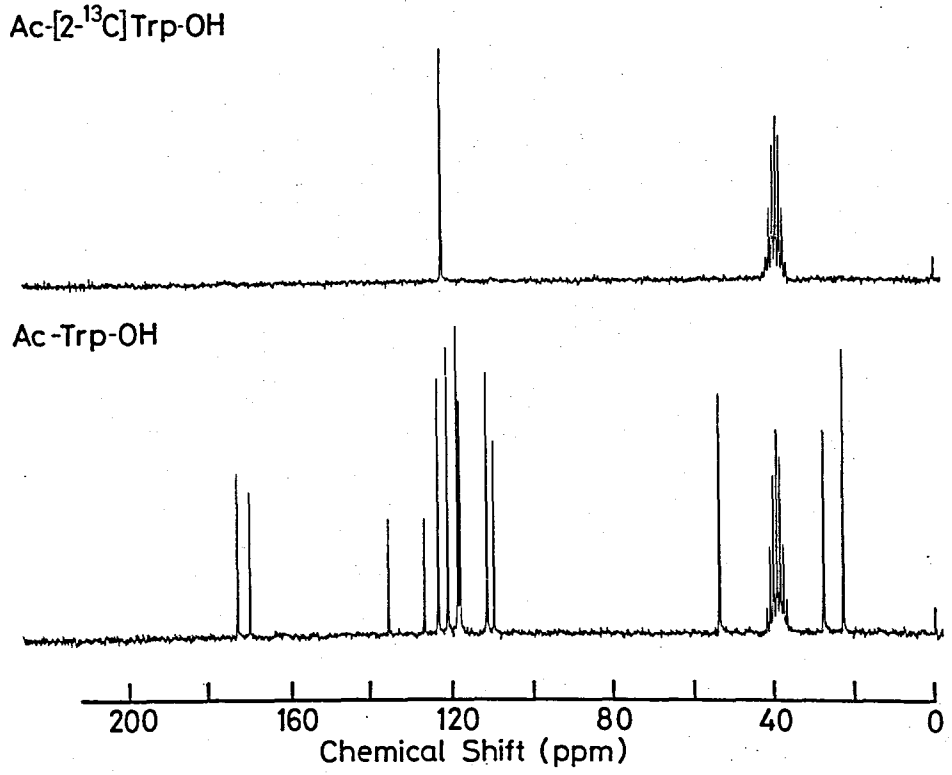


Fig. 1-7. ^{13}C-NMR spectra of Ac-[2-^{13}C]Trp-OH and Ac-Trp-OH. The spectra were recorded under complete proton-decoupled condition at 25.01 MHz in DMSO-d_6. Concentrations of the ^{13}C-enriched and unenriched samples were 2 mg/ml and 20 mg/ml, respectively. Scans: 3600 for the former and 4000 for the latter, with a recycle time of 1.5 s each.

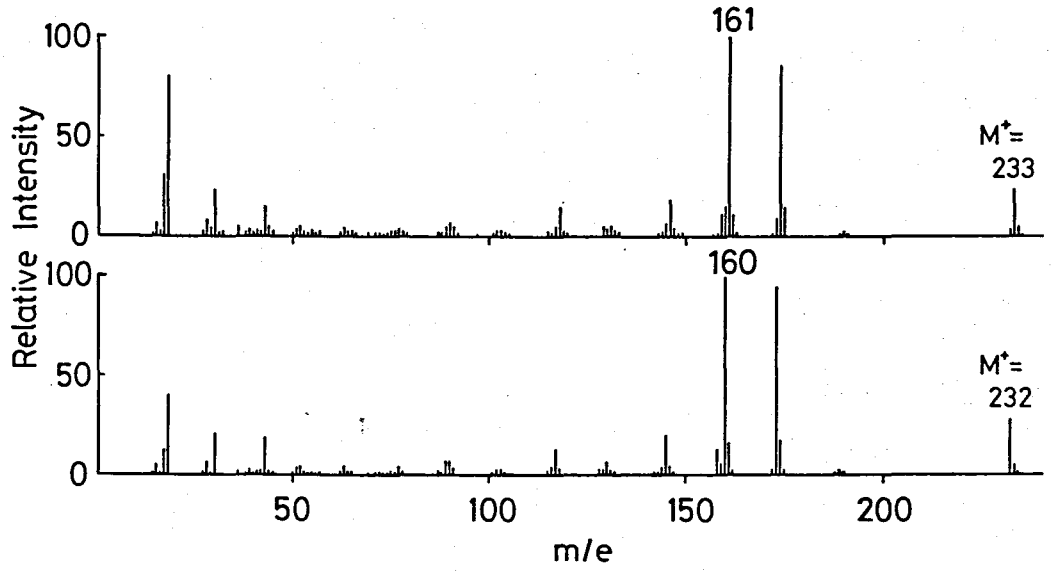


Fig. 1-8. EI-Mass spevtra of $^{13}C(90\%)$-enriched (upper) and unenriched (lower) melatonin. The base peaks at m/e = 161 (upper) and 160 (lower) correspond to M^+ - 72 due to the loss of $CH_2NHCOCH_3$ from the molecular ions.

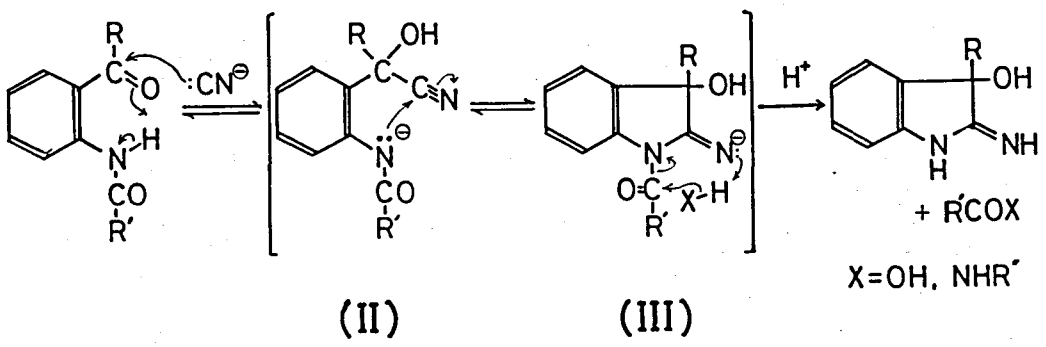


Fig. 1-9. A mechanism for the reaction of N-acylanthraniloyl compound with cyanide in the presence of a nucleophile (X-H).

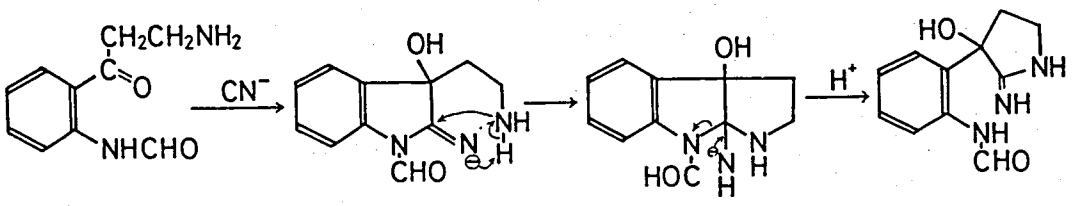


Fig. 1-10. The cyclization of 2-(N-formylanthraniloyl)ethylamine with cyanide to 2-iminopyrrolidin-3-ol.

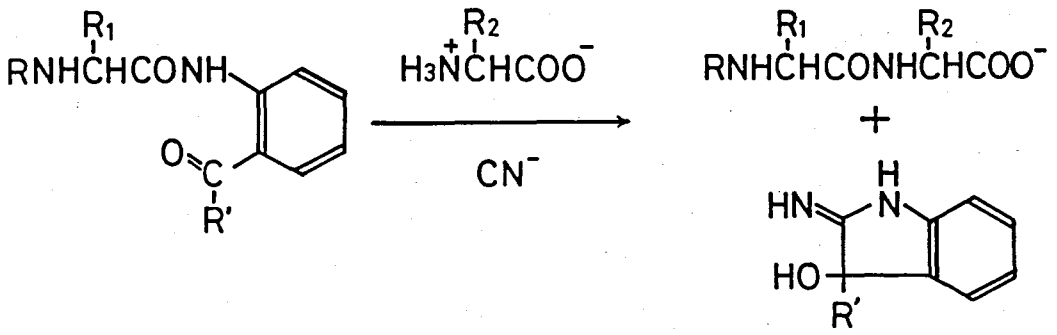


Fig. 1-11. The principle of the formation of peptide bond by the acyl-transfer reaction accompanied with the formation of 3H-indole.

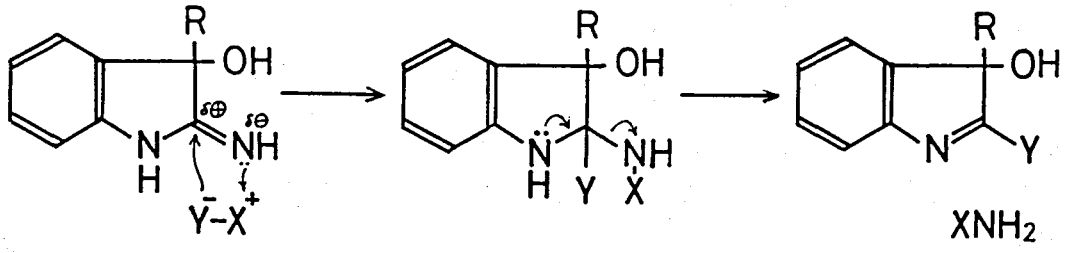


Fig. 1-12. Substitution of the C-2 "imino" group of 2-amino-3H-indol-3-ol with a nucleophile (Y^-).

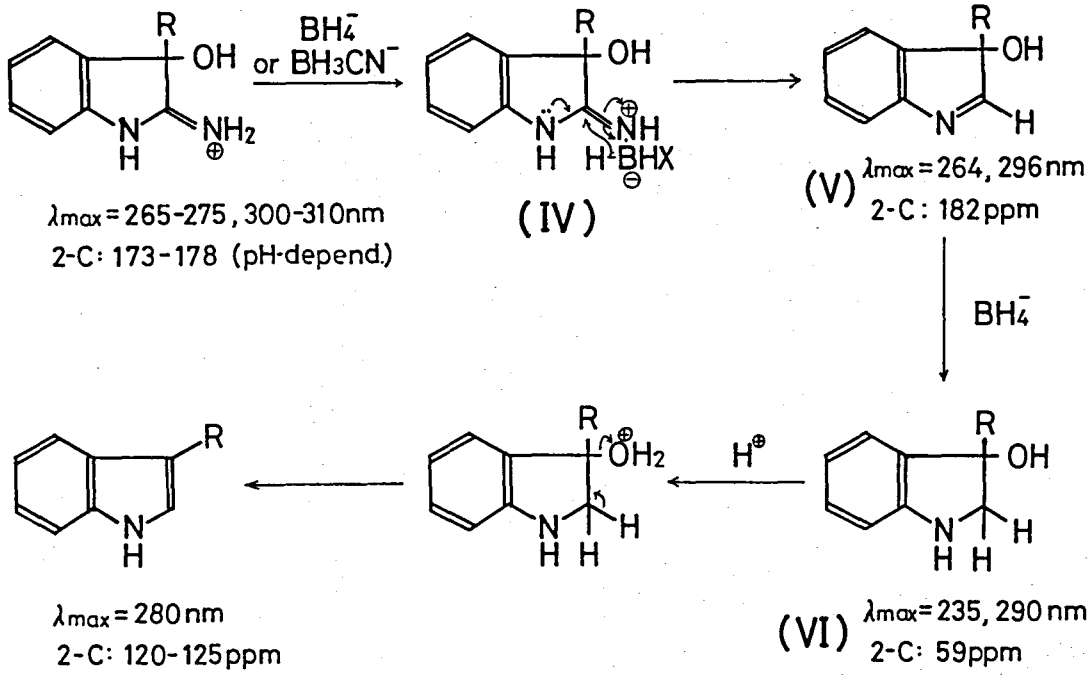


Fig. 1-13. A mechanism for the reduction of 2-amino-3H-indol-3-ols with hydrides. The intermediate (IV) should be read as a 2-aminoindoline derivative, although this Figure shows a transition state before the hydride attacks the C-2 carbon. "2-C" represents the chemical shift of the C-2 carbon resonance.

CHAPTER 2

^{13}C-Labeling of Lysoyzme at Tryptophan 62 and its ^{13}C Nuclear Magnetic Resonance Study

INTRODUCTION

Among many strategies for studying the structure-function relationships of proteins, the combined approach of isotope labeling technique and NMR spectroscopy has been recognized as a very effective means for the analysis of the behavior and the environment of a specific amino acid residue. Especially, for NMR measurements of nuclei such as ^{13}C and ^{15}N with low natural abundance, enrichment of a selected atom at a specified position in a protein molecule is advantageous. The information obtained from the isotopically enriched probe in the native protein must be unambiguous owing to unequivocal signal assignment.

Protein biosynthesis with isotopically labeled amino acids has been used for uniform labeling of a kind(s) of amino acid residues, but usually it is impossible to label the specified single residue by this method. Chemical modification utilizing the special reactivity of a selected residue among several ones is undoubtedly promising in this respect. However, such a chemical approach has been successfully used only for the labeling of methionyl S-methyl carbon (2).

The exchange reaction of the indole C-2 carbon described in Chapter 1 is expected to be applicable to proteins because of its mild reaction conditions. So we attempted to achieve the ^{13}C-labeling of hen egg-white lysozyme at tryptophan-62 (Trp-62), in order to establish a standard method to label the indole C-2 atoms of tryptophan residues in proteins.

It is worth noting here the today's aspects on the structure and function of lysozyme. Hen egg-white lysozyme (EC. 3.2.1.17) consists of 129 amino acid residues and holds both muramidase and chitinase activities. A pair of two acidic residues, Glu-35 and Asp-52, has been identified as catalytic groups. According to the mechanism proposed by Blake at al. (23), Glu-35 with the abnormally high pKa value acts as an acid to donate a proton to the glycosidic oxygen at the scissile bond of the substrate. The anion of Asp-52 with pKa 3.4 (35) stabilizes the resulted carbonium ion structure of the substrate by electrostatic interaction. On the surface of the lysozyme molecule, there are three essential tryptophan residues at positions 62, 63 and 108, which have been shown to be participated in the binding of substrate. In fact, modification of one of these residues results in the loss of enzyme activity. Among these three residues, Trp-62 has the most exposed side chain and is preferentially oxidized by ozone to N'-formylkynurenine (NFK). Lytic activity decreases to 30-40% upon oxidation of this residue, but increases to 80% by the deformylation of NFK to kynurenine (24). Trp-62 is also sensitive to N-bromosuccinimide, with which lysozyme loses enzyme activity significantly (25). From these experiments, the importance of the planar configuration involving the indole ring and the C-β atom was suggested. X-ray crystallography has shown that the indole NH group of Trp-62 and the sugar C-6 hydroxyl oxygen of substrate in subsite C are situated in the distance capable of hydrogen bonding

(23). However it has recently been shown that a substrate analogue, methyl 2-N-acetylamino-2,6-dideoxy-D-glucopyranoside, has the same binding power to lysozyme as its 6-hydroxy derivative (39).

The behavior of the side chain of Trp-62 has been also studied by ^{1}H- and ^{13}C-NMR spectroscopy. However, in these studies, the assignment of individual tryptophan resonances among many others has required pulse techniques using an NMR spectrometer operating at a high magnetic field (or high frequency $\geq$ 220 MHz) and sophisticated chemical modification techniques. Difficulties in the detection and the assignment of these signals limit an extensive application of NMR spectroscopy to the complex protein.

The ^{13}C-labeling of the protein at the specified position is one of the method to overcome these difficulties. As described in this manuscript, ^{13}C-labeled lysozyme at Trp-62 showed only the specific indole C-2 carbon resonance. Moreover, this resonance was titrated to a change of pH and the spin-lattice relaxation times were measured in the presence and absence of chitotriose.

This chapter deals with the ^{13}C-labeling of Trp-62 in hen egg-white lysozyme. In addition, the role and the environment of this residue in the structure and function of the active site are discussed, based on the information obtained by NMR spectroscopy using the ^{13}C-labeled lysozyme.

MATERIALS AND METHODS

[1] Materials: Hen egg-white lysozyme (6 times recrystallized, Seikagaku Kogyo, Tokyo) was used without further purification. Chitotriose (N-acetyl-D-glucosamine trimer) was prepared from chitin by the method of Raftery et al (26). Sephadex G-10 and CM-Sepharose CL-6B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

[2] Methods

i) Preparation of [2-^{13}C]Trp-62-Lysozyme.

a) Conversion of Trp-62 to N'-formylkynurenine

Lysozyme (1 g) was ozone-oxidized in water (100 ml) by the method of Kuroda et al (27). The oxidized protein (0.5 g) was chromatographed on a CM-Sepharose CL-6B column (2.2 x 120 cm) with a linear concentration gradient of NaCl from 0.25 M (1 liter) to 0.75 M (1 liter) in 0.05 M acetate buffer at pH 5.2. (Mizuno, K., unpublished method). NFK-62-lysozyme (OL-1 in Fig. 2-1) was isolated as lyophilizate after desalting on a Sephadex G-10 column (3.5 x 70 cm) with 0.1 M acetic acid. The yield was 380 mg.

b) Reaction of NFK-62-lysozyme with $^{13}CN^-$

NFK-62-lysozyme (200 mg) and 90% ^{13}C-enriched potassium cyanide (26 mg, Prochem.) were dissolved in 0.2 M glycine solution (10 ml). After the pH had been adjusted to 7.5-8.5 with acetic acid, the solution was stirred below 10°C for

1-2 days. To monitor the reaction, aliquots (50 µl each) were taken from the reaction mixture at times, diluted with water (3 ml) and measured the ultraviolet spectra. Figure 2-2 A shows the change of the spectra. At the end of the reaction, the ratio of absorbances at 290 to 250 nm (A_{290} / A_{250}) reaches to 1.36-1.40. The reaction was terminated by the additions of acetic acid to acidify the solution (pH 5.2), which was then directly chromatographed on a column of CM-Sepharose CL-6B (2.2 x 120 cm) under the same conditions described above. Two fractions of [2-^{13}C]AHT-62-lysozyme (CN-2 and CN-3, in Fig. 1B) were separately collected, desalted and lyophilized. Yields were 45 mg (CN-2) and 40 mg (CN-3).

c) Reduction of [2-^{13}C]AHT-62-lysozyme with $NaBH_4$ and formation of [2-^{13}C]Trp-62-lysozyme

[2-^{13}C]AHT-62-lysoyzme (CN-2 or CN-3, 30 mg) was dissolved in an aqueous 3M LiCl solution (3 ml) containing 0.2% EDTA·2Na. To this solution, were added the freshly prepared and ice-cold solution of $NaBH_4$ (3 mg/50 µl 3M LiCl) and 1-octanol (5 µl). The reaction was continued at 37°C for one hour. The reaction mixture was then acidified with 1 M HCl (100 µl) in order to decompose the excess $NaBH_4$ and to convert the reaction intermediate to tryptophan at the 62nd residue simultaneously. The slightly turbid solution was dialyzed using a #18/32 cellulose tubing (Visking Company) overnight against dilute HCl (pH 3-4) and then for several

hours against deionized water at 4°C. The dialyzate was chromatographed on MC-Sepharose CL-6B (1.1 x 44 cm) as described in legend to Fig. 2-5. [2-^{13}C]Trp-62-lysozyme thus obtained was 9 mg from CN-2 and 12 mg from CN-3.

ii) Preparation of [2,2-^{2}H,^{13}C]Trp-62-lysozyme

Lysozyme was converted to [2-^{13}C]AHT-62-lysozyme as described above. Sodium borodeuteride (99.8% $NaB^{2}H_4$ (Merck), 3 mg) in ice-cold 4M guanidium chloride solution (5 µl) was added to the 4 M guanidium chloride solution (3 ml) containing [2-^{13}C]AHT-62-lysozyme (CN-3, 30 mg) at 37°C. Upon addition of the reducing agent, hydrogen gas evolved vigorously and instantaneously. After an hour at 37°C, 1 M HCl (0.1 ml) was added to the solution, which was successively dialysed against 0.1 M HCl (five hours), deionized water (2 to 3 hours), cystine (10^{-4}M)-cycteine (10^{-3}M) solution (pH 8.5, overnight) and deionized water (2-3 hours) at 0°C. The dialyzate was chromatographed on a CM-Sepharose CL-6B column (1.1 x 44 cm) under the same conditions as described above. [2,2-^{2}H,^{13}C]Trp-62-lysozyme (9.6 mg, 32%) was obtained after desalting on Sephadex G-10 with 0.1 M acetic acid, followed by lyophilization.

iii) Enzyme assays

Lytic activity was measured by the method of Smolelis and Hartsell (28) toward the cell walls of Micrococcus lysodeikticus (Seikagaku Kogyo).

During the course of the preparation of [2-^{13}C]Trp-62-lysozyme, the specific lytic activity was measured for an aliquot (0.1 ml) of each fractions after chromatography on CM-Sepharose CL-6B at a constant protein concentration which was determined by the absorbance at 280 nm.

iv) Amino acid analyses

Amino acid analyses were performed with a Hitachi KLA-5 automatic amino acid analyzer after the hydrolysis of proteins with 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethy)-indole at 110°C for 24 hours. For the analysis of methionine sulfoxide, the sample was treated with cyanogen bromide in 70% formic acid at 37°C for 48 hours, lyophilized and then hydrolysed as above.

v) NMR measurements and pH titrations

^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL FX-200 NMR spectrometer operating at 199.5 and 50.10 MHz, respectively, in the pulse FT mode. For ^{1}H-NMR measurements, lysozyme was dissolved in 0.1 M $N^{2}H_4{}^{2}HCO_3$-$^{2}H_2O$ solution, incubated at 55°C for 30 minutes, and then lyophilized to remove the labile NH protons. Sample solutions were placed in 5 mm-tubes and chemical shifts were referred to the $H^{2}HO$ signal at 4.64 ppm determined by internal sodium 3-(trimethylsilyl)propionate-d_4 (Merck) at 0.0 ppm at neutral pHs at 37°C. ^{13}C-NMR measurements were conducted for $^{2}H_2O$ solution of labeled proteins placed in 10 mm-tubes. Chemical shifts were given in ppm from tetramethylsilane by referring to the value of internal

p-dioxane at 67.4 ppm. ^{13}C-NMR spectra of [2-^{13}C]AHT-62-lysozyme were also measured with a JEOL FX-100 NMR spectrometer at 25.01 MHz.

vi) ^{13}C-NMR titration

The ^{13}C-NMR titration was performed for [2-^{13}C]AHT-62-lysozyme at 1 mM concentration in $^{2}H_2O$ containing 0.1 M NaCl at 37°C. [2-^{13}C]Trp-62-lysozyme was similarly titrated at 0.4-0.5 mM concentrations. The pH of protein solution was adjusted with either 1 M ^{2}HCl or 1 M NaO^{2}H. The p^{2}H values (uncorrected pH meter readings) were measured with a Hitachi-Horiba pH meter F-7 equipped with a glass electrode (Ingold, #6030-40, 3.0 mmϕ) at 22-24°C. The chitotriose-^{13}C labeled (intact or AHT) lysozyme complex was titrated at concentrations of 1 mM for [2-^{13}C]AHT-62-lysozyme and 10 mM for chitotriose, or of 0.5 mM for [2-^{13}]Trp-62-lysozyme and 5 mM for chitotriose. The ionic strength of each sample solution was adjusted to 0.1 with NaCl. Titration curves obtained were analyzed either by the non-linear least squares program of one acid-base equilibrium for single sigmoidal curves or the program to search the least standard deviation from ideal values calculated from the equation shown below for double sigmoidal curves.

$$\delta_{obs} = \delta_0 + \frac{\Delta_1 \times 10^{(pH - pK_{a1})}}{1 + 10^{(pH - pK_{a1})}} + \frac{\Delta_2 \times 10^{(pH - pK_{a2})}}{1 + 10^{(pH - pK_{a2})}}$$

where δ_{obs} stands for the observed chemical shift, δ_0 is the value for the extreme at acidic pH, Δ_1 and Δ_2, are variations

of chemical shifts corresponding to the first and the second dissociation of groups with pK_{a1} and pK_{a2}, respectively.

vii) Measurements of spin-lattice relaxation times (T_1)

Spin-lattice relaxation times (T_1) of the enriched ^{13}C-2 carbon nuclei of Trp-62 and AHT-62 in hen egg-white lysozyme were measured by the inversion recovery method using a $(180°-\tau- 90°-T)_n$ pulse sequence, where τ is a variable interval between 180°- and 90°-pulses, and T is a constant pulse interval, which was set more than five times as long as the expected T_1 value. Samples were prepared following the same method as used for the ^{13}C-NMR titration experiments.

viii) Measurements of ultraviolet (UV) spectra

UV spectra were measured on a Beckman DB-GT grating spectrophotometer attached with a recorder (Unicorder U-125M. Beckman-Toshiba) and a Hitachi recording spectrometer EPS-3T. Qualz cells with path length of 1 cm were used.

ix) Affinity chromatography

Chitin-coated cellulose was prepared by the method of Imoto et al (29). The affinity column (1.6 x 7 cm) had been previously equilibrated with 0.1 M sodium phosphate buffer (pH 6.5) containing 0.1 M NaCl. The sample protein was dissolved in the same buffer and loaded onto the column. The column was thoroughly washed with the equilibration buffer to remove the unadsorbed protein. The active protein was then eluted with 0.1 M acetic acid.

x) The Bio-Rex-70 ion-exchange chromatography

Oligosaccharides bound to the active enzyme were removed by the chromatography on a column (1.2 x 43 cm) of Bio-Rex 70 (Bio-Rad) at pH 10.0 using the same buffer system according to the method of Imoto et al (29).

xi) Determination of free SH groups

The thiol (SH) groups liberated during the reduction of AHT with $NaBH_4$ were determined by the method of Ellman (30). A solution (10-100 µl) containing 10-50 nmol of the protein was acidified with 1M HCl (10 µl) to decompose the excess $NaBH_4$ and diluted with 0.05M tris-HCl buffer (3 ml) at pH 8.0. Twenty five µl of 0.01 M 5,5'-dithiobis-(2-nitrobenzoic acid) dissolved in 0.05 M phosphate buffer (pH 7.0) was added to this solution. The assay solution was allowed to stand for 10 minutes at 37°C, and measured the UV absorption at 412 nm.

RESULTS

[1] Preparation and characterization of [2-^{13}C]Trp-62-lysozyme.

i) Preparation of NFK-62-lysozyme.

The preferential ozone oxidation of Trp-62 to NFK was carried out by the method of Kuroda et al (27). The crude oxidation product was purified by ion-exchange chromatography on CM-Sepharose CL-6B, as shown in Fig. 2-1.

(Fig. 2-1)

In NFK-62-lysozyme eluted in fraction OL-1, the formation of one residue of NFK was confirmed spectrophotometrically. Amino acid analysis gave one mole of kynurenine and five moles of tryptophan as well as other 123 amino acid residues (Table 2-1).

Purified NFK-62-lysozyme was 35-40% active relative to native lysozyme for the lysis of Micrococcus lysodeikticus cell walls. A small portion of NFK at the 62nd residue was spontaneously deformylated to kynurenine (Kyn), by which lysozyme regained the lytic activity to 80% relative to the native enzyme. Kyn-62-lysozyme was somewhat retarded than NFK-62-lysozyme, but it was not easy to separate them each other completely by ion-exchange chromatography. However, further purification of NFK-62-lysozyme was not necessary because kynurenine was not reactive to cyanide and because the unreacted material could be easily removed by the next step.

ii) Preparation of diastereoisomers of AHT-62-lysozymes

The product analysis and the purification of isomers of AHT-62-lysozyme were routinely performed with ion-exchange chromatography on a CM-Sepharose CL-6B column after the reaction of NFK-62-lysozyme to cyanide. The chromatography was carried out under the same conditions as described above. The ion-exchange chromatogram of the reaction product is shown in Fig. 2-2B.

(Fig. 2-2B)

The desired reaction products, AHT-62-lysozyme isomers, were eluted in two close fractions, CN-2 and CN-3. They were identified by the ^{13}C-NMR spectroscopy of the ^{13}C-labeled samples. Protein eluted in those fractions gave characteristic ^{13}C signals at 175-177 ppm and ^{13}C-NMR titration curves with inflections at pH 6.0-6.7. The result of the ^{13}C-NMR titration study will be described later.

The difference between the proteins CN-2 and CN-3 was also found in UV spectra (Fig. 2-3)

(Fig. 2-3)

Lytic activities of proteins CN-2 and CN-3 were nearly 0% and 5-10%, respectively, relative to native lysozyme.

When these ^{13}C-labeled proteins were reduced with $NaBH_4$ as described later, the same ^{13}C-labeled active lysozyme indistinguishable by ^{13}C-NMR was obtained. These findings suggest that proteins CN-2 and CN-3 have the diastereomers of AHT-62.

Incorporation of the ^{13}C atom of cyanide was also detected in fraction CN-1, which showed ^{13}C signals at 180.5 and 181.5 ppm on ^{13}C-NMR spectra. Neither of signals had intrinsic dissociation character. These signals were eventually assigned to the indole C-2 carbon resonances of 2,3-dioxindolylalanine diastereoisomers at the 62nd residue. In fact, the two ^{13}C signals were observed at 181.5 and 180.5 ppm in hydrolysis (deamination) products of proteins CN-2 and CN-3, respectively. Amino acid analysis showed that the proteins in fraction CN-1 contained 0.3-0.5 moles of kynurenine (and/or NFK), suggesting that the formation of AHT was incomplete. The presence of Kyn-62-lysozyme in this fraction was also shown by relatively high lytic activity and characteristic UV absorption of kynurenine at 360 nm. The starting material, NFK-62-lysozyme, was eluted into this fraction.

Quantities of NFK-62-lysozyme unreacted and dioxindolylalanine-62-lysozyme reflected the formation and deamination of AHT-62-lysozyme. The amounts of these proteins did not decrease to less than 60% when the reaction was carried out at room temperature (15-30°C). However, the hydrolysis of AHT was suppressed below 10°C even at pH 8.0-8.5 and longer reaction period (2-3 days). The yield of protein CN-3 decreased significantly when the reaction was carried out at low pH and high temperature (20-30°C). This phenomenon is indicative of the much exposed state of the 62nd AHT residue in the protein CN-3, which is more susceptible to alkaline hydrolysis than protein CN-2.

The increased reactivity of AHT in protein CN-3 was also shown in the reduction with $NaBH_4$.

The product ratio of CN-2 to CN-3 was almost 1 : 1 when the hydrolysis of AHT was suppressed at low temperature. The ratio did not change by the addition of optically active amino acids. However, the participation of the added amino acid in the acceleration of the reaction was evident: the transfer of the formyl group of NFK to an amino acid was found when histidine was added. N^{α}-Formylhistidine and histidine were separated incidentally when the proteins in the reaction mixture were chromatographed on the CM-Sepharose CL-6B column. N^{α}-Formylhistidine was positive to the Pauli reagent and negative to ninhydrin on thin layer plates (silica gel, 1-butanol : acetic acid : water = 4 : 1 : 1 (v/v), Rf = 0.30, the material eluted faster than histidine showed the identical behavior with the authentic sample).

In this reaction, other side reactions such as the cleavage of disulfide bond by cyanide, as reported by Catsimpoolas and Wood (31), were not detected.

iii) Conversion of [2-^{13}C]AHT to [2-^{13}C]Trp at the 62nd residue of lysozyme

Reduction of the AHT residue was carried out with a 40-fold molar excess of $NaBH_4$ in 3M LiCl solution containing 0.2% EDTA disodium salt. As described in the preceding chapter, the addition of LiCl and EDTA was indispensable for the efficient re-

duction of AHT in 10^{-2}-10^{-3}M protein solutions. The reaction medium was initially adjusted to slightly acidic (pH 4-5) at 37°C. Under these conditions, the thiol groups liberated during the reaction were less than 0.2 moles per mole of protein as determined by the method of Ellman (30). The reduction of disulfide bond was increased when 3 M guanidium chloride was used in place of 3 M LiCl, as shown in Fig. 2-4.

(Fig. 2-4)

The reaction mixture was acidified to regenerate tryptophan and analyzed either by the ion-exchange chromatography on CM-Sepharose CL-6B or by the affinity chromatography on a chitin-coated cellulose column. Fig. 2-5 shows the chromatogram on CM-Sepharose CL-6B. The elution profiles of crude ozone-oxidized lysozyme and crude AHT-62-lysozyme are also presented.

(Fig. 2-5)

Fig. 2-5C clearly shows that the protein in fraction RL-1 possesses full lytic activity and the same retention volume as native lysozyme (see Fig. 2-5A).

The yields of fully active protein from proteins CN-2 and CN-3 were different. Fig. 2-6 shows the elution pattern of the reaction products derived from proteins CN-2 and CN-3 on chitin-coated cellulose column.

(Fig. 2-6)

The adsorbed protein was further purified with the ion-exchange chromatography on Bio-Rex 70, as shown in Fig. 2-7.

(Fig. 2-7)

The yield of [2-^{13}C]Trp-62-lysozyme was always higher for protein CN-3 than for protein CN-2.

The active protein derived from proteins CN-2 and CN-3 by reduction was identified as intact lysozyme in terms of UV spectra and amino acid compositions as well as lytic activities and chromatographic behaviors. Fig. 2-8 shows the UV spectra of native and ^{13}C-labeled lysozyme.

(Fig. 2-8)

Table 2-1 lists amino acid compositions of regenerated lysozyme and other lysozyme derivatives obtained in the ^{13}C-labeling reaction.

(Table 2-1)

The identity in the tertiary structure between native and the ^{13}C-labeled lysozyme was confirmed by ^{1}H-NMR spectroscopy (Fig. 2-9).

(Fig. 2-9)

When the ^{1}H-NMR spectra of native and 90% ^{13}C-enriched lysozyme were carefully compared with each other in the region of aromatic protons, a difference was found in the shape of the spectrum.

(Fig. 2-10). The intensity of the proton signal at 7.01ppm in the ^{13}C-enriched lysozyme considerably decreased compared with that of the native protein, while a new signal appeared 90.4Hz upfield. The counterpart of the split signal was detected at the corresponding downfield position in the overlapping region of proton signals.

(Fig. 2-10)

Finally, the homogeneity of the ^{13}C-labeled protein and the formation of [2-^{13}C]Trp-62 were examined by ^{13}C-NMR spectroscopy. Fig. 2-11 shows the ^{13}C-NMR spectra of ^{13}C-labeled lysozyme.

(Fig. 2-11)

Enriched ^{13}C gave a single resonance at 125.1ppm in $^{2}H_2O$ at p^{2}H 4.7 under the fully proton decoupled condition and a doublet with a ^{13}C-^{1}H coupling constant of 180.7Hz under the gated proton-decoupled condition. The ^{13}C-^{1}H coupling constant of 180.7Hz obtained by the ^{1}H- and ^{13}C-NMR spectra were precisely coincided with each other and this value was in good agreement with the value (182Hz) obtained for the corresponding proton signal of 3-([2-^{13}C]indol-3-yl)propionic acid (16).
The C-2 proton and carbon resonances of Trp-62 were further correlated by the selective irradiation of the proton signal at 7.01 ppm. Fig. 2-12A shows the selectively proton-decoupled ^{13}C-NMR spectrum of [2-^{13}C]Trp-62-lysozyme.

(Fig. 2-12)

Fig. 2-12B shows the non-decoupled ^{13}C-NMR spectrum of [2,2-^{2}H,-^{13}C]Trp-62-lysozyme to demonstrate the effect of the double labeling of Trp-62 with ^{2}H and ^{13}C at the indole C-2 position. By the incorporation of ^{2}H, the ^{13}C resonance was shifted about 0.2 ppm upfield relative to the ^{13}C atom bearing ^{1}H, probably by the deuterium isotope effect.

[2] ^{13}C-NMR study of [2-^{13}C]Trp-62-lysozyme and its derivative

i) ^{13}C-NMR titration.

The ^{13}C-NMR titration study and the measurements of spin-lattice relaxation time were carried out for [2-^{13}C]Trp-62-lysozyme and diastereoisomers of [2-^{13}C]AHT-62-lysozyme. Fig. 2-13 shows the ^{13}C-NMR titration curves for the enriched C-2 carbon resonances of AHT residues. The titration curve for the corresponding C-2 carbon signal of Ac-AHT-NH_2 is also shown in this Figure.

(Fig. 2-13)

From these titrations, apparent pKa values were estimated to be 6.7 and 6.1 for proteins CN-2 and CN-3, respectively. The protein CN-3 which had the lytic activity of 5-10% relative to native lysozyme showed an additional inflection point at p^{2}H 3.4 in the titration curve, whereas this inflection disappeared in the presence of chitotriose, a competitive inhibitor of lysozyme. In

this case, the inhibitor exerted little influence on the pKa value of the AHT residue itself.

In contrast, the C-2 carbon of Trp-62 showed different behavior to the change of pH in the presence and absence of the inhibitor, as shown in Fig. 2-14.

(Fig. 2-14)

When [2-^{13}C]Trp-62-lysozyme was titrated, the influence of a dissociation equilibrium of a group with pKa 3.8 was observed. In the presence of the inhibitor, however, the C-2 resonance shifted to upfield and another inflection point appeared at p^{2}H 6.5 in addition to that at p^{2}H 3.8.

ii) Spin-lattice relaxation time (T_1)

The spin-lattice relaxation time measurements were carried out for [2-^{13}C]AHT- and [2-^{13}C]Trp-62-lysozymes in the presence and absence of chitotriose.

Fig. 2-15 shows the stacked spectra measured for [2-^{13}C]AHT-62-lysozyme in the presence of the inhibitor.

(Fig. 2-15)

The plot of τ versus $-\ln[(I_\tau - I_\infty)/I_\infty]$ is shown in Fig. 2-16.

(Fig. 2-16)

The T_1 values were calculated from the slopes. The T_1 values of the indole C-2 carbon of AHT-62 were 900 ms and 530 ms in the presence and absence of chitotriose, respectively.

Fig. 2-17 shows the ^{13}C-NMR spectra of [2-^{13}C]Trp-62-lysozyme for several τ values in the presence of a 10-fold molar excess of the inhibitor.

(Fig. 2-17)

The intensities of signals and the corresponding τ values were plotted in the same manner as above. Fig. 2-18 shows the semi-logarithmic plots.

(Fig. 2-18)

The T_1 value determined for the C-2 carbon of Trp-62 was 150ms for free lysozyme and 100ms for its complex with chitotriose.

DISCUSSION

[1] Procedure for the ^{13}C-labeling of lysozyme

The method for the isotope lableing of the indole C-2 atoms was applied to Trp-62 in hen egg-white lysozyme. The isolation of fully active ^{13}C-labeled lysozyme indicates that this ^{13}C-labeling reaction is highly selective to the target residues in the protein. Scope and limitation of this method as the specific isotope labeling of tryptophan in proteins will be discussed together with some comments on individual reactions employed.

i) Ozone oxidation of Trp-62 in lysozyme

In hen egg-white lysozyme, Trp-62 was oxidized in an aqueous solution with ozone according to the method of Kuroda et al (27). Ozone concentrations were 0.5-1 μmol/min, or 0.002-0.005% O_3/O_2 at 0-5°C, but the reaction with much higher, or even lower, ozone concentrations caused non-specific oxidation of tryptophan; Trp-108 has been shown to be modified as well as Trp-62 (32). For the present experiment, a limited amount of ozone (0.75-0.85 mol/mol of lysozyme) was used at concentrations of 0.7-1.2 μmol/min. and the oxidation product was purified with ion-exchange chromatography. As shown in Fig. 2-1, the product was a mixture of NFK-62-lysozyme (35-40%), unoxidized lysozyme (20-25%) and excessively oxdized proteins (40-45%). Therefore the selectivity of the

^{13}C-labeling reaction is primarily based on the selectivity of the reaction to convert tryptophan to N'-formylkynurenine. It is possible to label a tryptophan residue (s) other than Trp-62 if the conditions to modify those residues to N'-formylkynurenine selectively and the method to purify the modified protein are established.

ii) Formation and isolation of AHT-62-lysozyme isomers.

The aim of preliminary experiments was to establish the condition to convert NFK to AHT effectively without the deamination of the latter compound. Although the rates of the formation and deamination of AHT have apparently the same pH and temperature dependencies, the addition of alkylammonium salt as an acyl acceptor to reaction media increased the formation of AHT. As described in Chapter 1, the alkylammonium salt including α-amino acids was very effective and the AHT formation indeed occurred considerably even at around 0°C where the deamination of AHT is suppressed. The use of higher concentrations of cyanide was another choice, but it was not suited for this reaction because of the reactivity of cyanide to disulfide linkages. No modification of disulfides was detected under the present conditions; four cystine residues were in fact determined by amino acid analysis in AHT-62-lysozyme. Additionally, the use of a large excess of ^{13}C-labeled cyanide is not economical.

For the isolation of AHT-62-lysozyme, the cation-exchange chromatography proved effective because the protein had an

additional positive charge at the AHT residue in acidic media. In fact, the proteins containing AHT were eluted in the retarded fractions relative to NFK-62-lysozyme and even to native lysozyme.

Among several criteria to identify the AHT residue, ^{13}C-NMR spectroscopy was effective for the ^{13}C-enriched protein as well as the identification of the tryptophan residue formed by reduction with sodium borohydride.

UV spectroscopy and amino acid analysis were not helpful to distinguish AHT from dioxindolylalanine, the deamination product. AHT and dioxindolylalanine were decomposed to unidentified ninhydrin negative compound(s) during acid hydrolysis.

It was found that proteins CN-2 and CN-3 (in Fig. 2-2B) had the enriched C-2 carbon signal due to AHT's at reasonable resonance positions around 175 ppm and the pKa values of 6.7 and 6.1, respectively. The validity of the assignment of proteins CN-2 and CN-3 to two lysozyme derivatives containing the diastereoisomeric AHT residues at positions 62 was shown by the fact that both AHT proteins yielded fully active lysozyme, which possesses the enriched ^{13}C atom at Trp-62 and the same tertiary structure as the native protein. The separation of these two isomeric species of AHT-62-lysozyme by chromatography on CM-Sepharose CL-6B was surprising when a difference only in the configuration at the side chain of residue 62 in the 129 amino acid residues of lysozyme is con-

sidered. It is reasonable to suppose that the ^{13}C-NMR titration data of proteins CN-2 and CN-3 may explain their different chromatographic behavior on the cation exchanger column. However, protein CN-3 in which AHT-62 has a lower pKa value than that in protein CN-2 was eluted more slowly than the latter protein. It is likely that the AHT residue is interacting with a positive charge(s), probably the guanidino group of the adjacent Arg-61, and that the mode of electrostatic interaction differs from each other in each diastereoisomers of the AHT residue. Therefore, that protein CN-3 is more basic than CN-2 suggests that the interaction of AHT residue with Arg-61 is stronger in protein CN-3 than in protein CN-2, giving rise to the increase of the basicity of the adjacent guanidino group in the former protein. The almost equimolar formation of these isomeric proteins is probably due to a considerably high mobility of the side chain at the 62nd NFK residue. If not, the reaction would have more or less proceeded in an enantio-selective manner owing to essentially the chiral nature of the protein matrix around the target residue.

iii) Reduction of [2-^{13}C]AHT-62 and characterization of [2-^{13}C]-Trp-62-lysozyme

Reduction of AHT-62-lysozyme with $NaBH_4$ occurred in the absence of denaturant without any difficulties. This may be due to the highly exposed state of residue 62 on the surface of the protein molecule. Disulfide bonds are in general

reactive to borohydride, but, in this case, all of the four disulfide bonds buried inside the molecule were unreactive. For proteins with low solubility in 3M LiCl, guanidium chloride could be used in place of the alkali halide when they are reversibly refolded from the denatured states.

The 3M LiCl solution containing the lysozyme derivative involved 3-hydroxyindoline intermediate became slightly turbid upon acidification and then clear during dialysis. The cause of the turbidity is unclear.

Regenerated lysozyme was readily isolated from the reaction products either by the CM-Sepharose CL-6B chromatography or by affinity chromatography on a chitin coated cellulose column. As shown in Fig. 2-5C, a protein possessing a relatively high lytic activity was eluted faster than native lysozyme. From the elution position, this protein is perhaps deaminated lysozyme which is adsorbed on the affinity column. The regenerated lysozyme was eluted from the affinity column together with oligosaccharides of N-acetyl-D-glucosamine which were attached noncovalently to the cellulose support as affinity ligands. Therefore, this protein sample must have been further purified by the chromatography on Bio-Rex 70. The CM-Sepharose CL-6B chromatography was unsuccessful because the protein-oligosaccharide complex adsorbed tightly on the ion-exchanger resin under the present conditions. Thus, it is concluded that the regenerated lysozyme has enzyme activity and ionic property identical to native lysozyme.

The presence of the 62nd tryptophan residue in regenerated

lysozyme was apparent from its native character in structure and function. The intactness of the residue other than the 62nd residue during a series of reactions for ^{13}C-labeling was confirmed by amino acid analysis. Especially the oxidation of methionine to the sulfoxide was the most suspected side reaction, but the experiments clarified the absence of methionine sulfoxide in regenerated lysozyme. The folding of regenerated lysozyme to native conformation was proved by ^{1}H-NMR spectroscopy, which clearly showed that native lysozyme and [2-^{13}C]Trp-62-lysozyme had the identical fine structures in their spectra including the signals up to - 1 ppm. Among the methyl proton signals, the one observed at 0 ppm in both proteins is important. This signal has been assigned to the S-methyl protons of Met-105 by Campbell et al. (33) who have concluded that the ring current effect of Trp-108 might shift the S-methyl resonance to such a high-field. The present finding indicates that the spatial arrangement of these two residues is preserved in ^{13}C-labeled lysozyme and that Met-105 is intact. Naturally, the same are described for all other signals. The minor but meaningful difference in ^{1}H-NMR spectra between native and [2-^{13}C]Trp-62-lysozyme concerns with the indole C-2 proton signal of residue 62 because of the spin coupling with the adjacent enriched ^{13}C nucleus in the labeled protein. This is the signal at 7.01 ppm, which has been assigned to the C-2 proton of Trp-62 by Cassels et al.(34). It should be noted that the ^{13}C-labeling of the indole C-2 atom was useful not only for ^{13}C-NMR spectroscopy, but also

for the signal assignment in the ^{1}H-NMR study. The assignment of ^{1}H signals using the characteristic ^{13}C-^{1}H coupling at a specified position in a protein is unequivocal and reliable since the isotope labeling does not perturb the native structure of the protein. Therefore, the result of the present experiment should be a proof that the signal assignment proposed by Cassels et al. is correct.

The scalar coupling of this proton signal with the enriched ^{13}C nucleus was directly evidenced by the double resonance experiment in which the proton signal at 7.01 ppm was selectively irradiated. The resulting ^{13}C-NMR spectrum showed a singlet due to the enriched ^{13}C-signal. Even a completely proton-decoupled spectrum gave a single ^{13}C-resonance which shifted homogeneously according to the change of pH. This fact indicates that the ^{13}C atom was incorporated in lysozyme at Trp-62 specifically.

The indole C-2 atoms of Trp-62 were successfully labeled with ^{2}H and ^{13}C as evidenced by the proton-coupled ^{13}C-NMR spectrum which showed the slightly broad singlet at 124.8 ppm. Theoretically, the ^{13}C-signal should be split to a triplet due to the ^{2}H-^{13}C coupling. However, the broadening of the ^{13}C signal probably owing to the chemical shift anisotropy under the considerably high (47 kG) magnetic field may prevent the resolution of the triplet signal.

The present ^{13}C-labeling reaction was successfully applied to lysozyme and [2-^{13}C]Trp-62-lysozyme was prepared. The isotope-

labeling at tryptophan can be accomplished with ^{14}C or/and ^{3}H and may generally be applicable to any proteins because of the mildness and the high selectivity of the reactions involved.

[2] ^{13}C-NMR study of intact and modified lysozymes labeled with ^{13}C

As shown by the ^{13}C-NMR titration of [2-^{13}C]Trp-62-lysozyme in the presence of chitotriose, the indole C-2 carbon signal of Trp-62 was associated with dissociation equilibria of two independent ionizable groups having the apparent pKa values of 3.8-3.9 and 6.5. By referring these pKa values to those determined for ionizable groups of hen egg-white lysozyme (35 , 36), it is reasonable that the acidic and neutral groups are assigned to the carboxyl groups of Asp-52 and Glu-35, respectively. Both groups have been proposed to constitute the catalytic site of this enzyme and to participate directly in interaction with substrate. Table 2-2 lists the pKa values presented for these two groups.

(Table 2-2)

Titration data showed that Asp-52 had an influence on the 62nd tryptophan residue regardless of the presence and absence of chitotriose. The effect of Glu-35 was detected only for the lysozyme-chitotriose complex. According to the illustration drawn by X-ray crystallographic data by Blake et al. (23), a pair of Glu-35 and Asp-52 exists at each side in the active site cleft and the two indole rings of Trp-62 and Trp-63 are situ-

ated closely at the same side of Asp-52. However, the direct contact of Asp-52 and these indole rings is obstracted by the side chain amide group of Asn-59 situated between them. Therefore, it appears that the interaction between Trp-62 and Asp-52 is mediated by Asn-59 or by Asn-59 and Trp-63. The direct interaction across the cleft between Trp-62 and Glu-35 is unlikely.

Under these circumstances, it is likely that the orientation of the indole ring of either Trp-62 or Trp-63 is perturbed by the ionization of Asp-52 via Asn-59. If this is the case, the ionization of Asp-52 induces the movement of the bulky side chain of Trp-62 or 63, resulting in the change of relative spatial arrangement of both the 62nd and the 63rd residues. The proximity of the two indole rings at positions 62 and 63 induces the ring-current shift of their ^{1}H and ^{13}C resonances mutually. The shift is probably significant because the effect is inversely proportional to the 3rd power of the distance between these residues.

It is certain that the mechanism by which Asp-52 perturbs the conformation of Trp-62 is also operating in the lysozyme-chitotriose complex. The upfield shift of the titration curve in that complex is due to a change in the relative arrangement of two indole rings of Trp-62 and Trp-63 by inhibitor binding. X-ray crystallographic analysis has shown that the indole ring of Trp-62 moves about 0.75Å to narrow the active site cleft when chitotriose binds to lysozyme in sub-site A to C (23). The binding mode of chitotriose to lysozyme is illustrated in Fig. 2-19.

(Fig. 2-19)

Although two domains involving Asp-52 and Glu-35 are separated by the active site cleft, they can be connected by the insertion of a substrate. Perkins et al. (37) have studied the ^{1}H- and ^{13}C-NMR titrations of the bound substrate analogues and observed the influence of both Glu-35 and Asp-52 on the titration curves for the 2-acetamido proton resonances. Apparent pKa values of 6.4 $\pm$ 0.1 and 3.9 $\pm$ 0.3 were determined for Glu-35 and Asp-52, respectively. These values are in good agreement with those obtained in the present work. It is interesting that, according to the X-ray crystallographic data by Perkins et al.(38), the acetamido methyl protons of β-methyl N-acetyl-2-deoxy-2-amino-D-glucopyranoside are on an average 0.26 nm above the Trp-108 indole ring plane. The methyl protons may be influenced significantly by the ring current of Trp-108. Accordingly, the long range interaction between Trp-62 and Glu-35 observed in the present study may occur through Trp-108 and the chitotriose molecule which has numerous contacts with both Trp-62 and Trp-108. Trp-108 is located close to the carboxyl group of Glu-35.

It is expected that not only the active site but also the bound substrate molecule undergo subtle pH dependent conformational changes following the dissociation equilibria of the two catalytic groups, Asp-52 and Glu-35. These conformational changes may be closely related to the modulation of catalytic function, especially

the binding function at the active site of lysozyme. Therefore, it is reasonable that the binding constant (K_{ES}) of lysozyme to chitotriose principally reflects the ionization behavior of the catalytic groups, as demonstrated by the circular dichroism (CD) study by Kuramitsu at al. (35). The pH-K_{ES} profile and the ^{13}C-NMR titration data reflect the same phenomena. Recently, kinetic study using 4-methylumbelliferyl chitotriose (36) has provided evidence supporting the above conclusion.

The chemical shift of the C-2 carbon of [2-^{13}C]AHT-62-lysozyme (CN-3) depended on pH in a different manner from above. The influence of Asp-52 on the AHT residue was observed in the absence of chitotriose similar to the case of native lysozyme, while this influence disappeared in the presence of the inhibitor. The titration curve of the ^{13}C-signal of ^{13}C-enriched protein CN-2, which exerted no lytic activity, showed no such response to the external dissociation equilibria. These results imply the importance of the flexibility or the sensitivity of the 62nd residue to the change of the environment.

In this respect, the T_1 values for the indole C-2 carbon of Trp-62 and AHT-62 were measured in order to compare the motions of these indole rings in the presence and absence of chitotriose. The measured T_1 value at 47 kG for the C-2 carbon of Trp-62 in the absence of chitotriose was 150 ms, which corresponded to the correlation time (τ_R) of 0.5-1 ns as estimated from the diagram of Doddrell et al. (40). The τ_R value is somewhat smaller than the reported value ($\tau_R \simeq 10$ ns)

of hen egg-white lysozyme (41). The difference may be attributed to the free rotational motion of the indole ring. In fact, silmilar T_1 values were obtained for tryptophan residues in small peptides (42 , 43). On the other hand, the T_1 value for the same carbon in the presence of chitotriose was 100 ms corresponding to τ_R = 2-3 ns, which suggests that the motion of Trp-62 is restricted by inhibitor binding. On the contrary, the AHT residue of protein CN-3 appeared to move or rotate more freely in the presence of chitotriose than in the absence. In fact, the T_1 values at 23.5 kG for the indole C-2 carbon of AHT-62 were 900 ms in the presence and 530 ms in the absence of the inhibitor. It is difficult to correlate these values with τ_R because the C-2 carbon of AHT bears no proton but two nitrogen atoms*. However, if the total motion of the AHT residue and the protein-chitotriose complex is assumed to be represented by the τ_R value of 0.5-1 ns which is estimated for free intact lysozyme, the measured T_1 value of 900 ms is in fairly good agreement with the T_1 value estimated from the diagram by Norton et al. (44). The T_1 value for free AHT-lysozyme suggests a relatively restricted motion of the AHT residue compared with the complex with chitotriose. Perhaps, the tetrahedral configuration of the C-3 carbon of AHT

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*The observed T_1 value is given by

$$1/T_1 = 1/T_1^D + 1/T_1^{CAS}$$

where T_1^D and T_1^{CAS} stand for dipole spin-lattice relaxation and chemical shift anisopropy relaxation respectively (45). For protonated carbons in a large molecule like lysozyme, the ^{13}C-^1H dipole interaction predominates and thus $T_1 \simeq T_1^D$. However, for non-protonated carbons like this one, T_1^{CAS} should be taken into account as well as T_1^D. In addition, the dipole interaction of ^{13}C-^{14}N should be involved in T_1^D.

may force the indole ring to situate at a locus unfavourble to catalytic function. It is likely that inhibitor binding indûces a change in the conformation of AHT-62 and the surrounding residues, resulting in the increase of the freedom in the motion of the AHT residue. The release of the AHT residue from such a restricted state(s) may correspond to the disappearance of the influence of Asp-52 in the titration curve.

In AHT-62-lysozyme (CN-3), a decrease in enzyme activity may be attributed to the perturbed conformation around the 62nd residue which plays essential roles in function of lysozyme by participating in the binding of substrate and by cooperating with the catalytic groups during catalysis.

[3] Meanings and advantages of isotope labeling technique

i) Information

It is worth noting that the important information specific for Trp-62 could be obtained first by the experiment using the ^{13}C-labeled species of native lysozyme. Norton and Allerhand (46) have conducted the assignment and the pH-titrations of the six C-3 (C^{γ}) carbon resonances of the tryptophan residues of lysozyme at 9 mM solution by natural abundance ^{13}C-NMR spectroscopy. They have found that the titration curve of Trp-62 has the inflection at pH 6.1 which corresponds to the pKa value of Glu-35. They have concluded that the interaction between these two residues is caused by the self-association of lysozyme at relatively high concentrations. Therefore, in natural abundance

^{13}C-NMR spectroscopy, it is difficult to study the interaction of lysozyme with chitotriose. The same experimental limitation also exists in ^{1}H-NMR spectroscopy for 5 mM lysozyme solutions reported by Cassels et al.(34). In the present study, the protein concentration was 0.5-1 mM and self-association seems unlikely at 0.1 ionic strength in the whole pH range examined. With 90% ^{13}C-enrichement, these concentrations are comparable with the 45-90 mM solutions of unlabeled lysozyme. Hence, the presence of the 10-fold excess chitotriose did not interfere the measurement of NMR spectra. The measurement of T_1 value for a single aromatic carbon resonance in a protein in the present study is the first example.

To elucidate the mechanism of the active site associated with catalysis, it is necessary to detect and to analyze the subtle change occurred cooperatively in the enzyme and the substrate. As described hitherto, it appears that any groups comprising the active site as well as those participating in the binding of substrate take parts in the constitution of catalytic function. The present ^{13}C-labeling method must make a significant contribution to the investigation along this line and, as a consequence, to the understanding of mechanism of the enzyme as a very efficient catalyst.

2) Methodology

As the method for the introduction of isotopic probes to the exactly intact protein, biosynthetic (47 - 48), semi-synthetic (chemical: 49; and enzymic: 50) and chemical

modification (2, 51,52) approaches have been reported. Among these techniques, the pH-dependent C-2 hydrogen-tritium (deuterium) exchange reaction for histidine residues, which was established in our laboratory (1), is the most elegant method. This method has been successfully applied for the investigation of enzyme catalytic sites where the histidine residue is often found. When these approaches are compared with respect to (i) the specificity of labeling, (ii) application to proteins, and (iii) the isotope to be introduced, chemical modification is the most versatile approach. In principle, the approach utilizing semisynthesis of proteins is promising, but at present it is premature because of the lack of the established experimental techniques.

In contrast, chemical modification of proteins now holds the accumulated knowledge concerning their reactivities to a variety of chemical reagents and proteolytic enzymes. Based on this consideration, it is possible to design a reaction(s) to satisfy the requirements described above. However, the chemically multi-functional character of proteins restricts the use of chemicals and the target residues to be labeled. The specific ^{13}C-labeling has been successfully accomplished for tryptophan and methionine (2) in proteins.

I believe that the investigation utilizing this labeling method to proteins will contribute not only to the more profound understanding of their structures and functions but also to open a new area of protein chemistry in the future.

Table 2-1. Amino acid compositions of regenerated lysozyme and lysozyme derivatives in which the 62nd residues are modified to NFK and AHT.

Amino acid	OL-1 (NFK-62)	CN-2 (AHT-62)	CN-3 (AHT-62)	Regenerated (Trp-62)
Lys (6)	6.00	5.87	5.84	5.98
His (1)	0.97	0.96	1.03	0.97
Arg (11)	11.27	11.01	10.99	10.89
Trp (6)	5.06	4.46	4.45	5.51
Kyn*[1]	0.91	±	-	-
Asp (21)	21.02	21.82	21.70	20.72
Thr (7)	6.55	7.28	7.23	6.56
Ser (10)	8.95	10.47	10.47	8.48
Glu (5)	4.65	4.88	4.84	4.95
Pro (2)	1.87	2.10	2.25	1.92
Gly (12)	11.90	12.78	12.81	12.20
Ala (12)	12.02	13.04	13.07	12.35
Cys*[3] (4)	4.05	3.60	3.57	3.72
Val (6)	5.64	4.37	4.50	6.17
Met (2)	1.74	1.74	1.87	1.84*[2]
Ile (6)	5.72	4.47	4.56	5.67
Leu (8)	(8.00)	(8.00)	(8.00)	(8.00)
Tyr (3)	2.94	3.12	3.22	2.84
Phe (3)	2.94	2.91	2.87	2.83

*[1] Kynurenine. *[2] Reacted with BrCN almost completely indicating no methionine sulfoxide is involved.
(The values for Trp and Cys were corrected by the factor of 1/0.85 for decomposition during hydrolysis.) *3 Cystine.

Table 2-2. The pKa values for Glu-35 and Asp-52 in hen egg-white lysozyme and its complex with chitotriose.

State	Glu-35	Asp-52	Method
Free	6.1	3.4	CD*2
Bound*1	6.5	3.4	
Free	6.20	3.60	Kinetics*3
Bound	6.55	3.95	
Free	-	3.88	^{13}C-NMR*4
Bound	6.51	3.79	

*1 Lysozyme-chitotriose complex *2 By Kuramitsu et al. (35).

*3 By Yang et al. (36). *4 Present study.

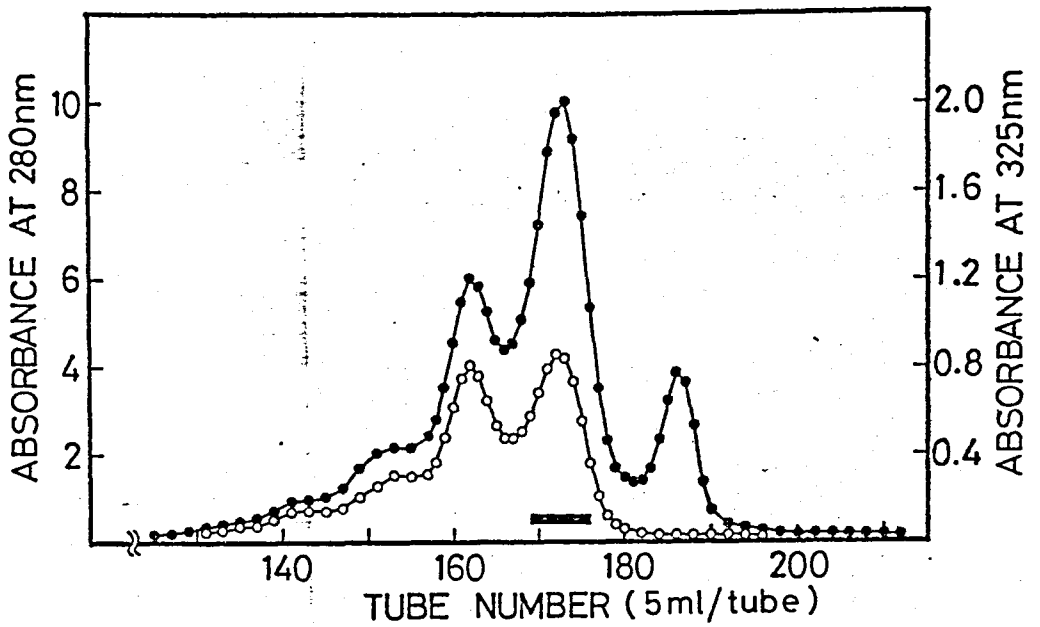


Fig. 2-1. Chromatography of crude oxidized lysozyme on CM-Sepharose CL-6B. Crude ozonization product of lysozyme (500 mg, after lyophilization) was applied to a CM-Sepharose CL-6B column (2.2 x 120 cm) and chromatographed with a linear concentration gradient of NaCl from 0.25M (1 liter) to 0.75M (1 liter) in 0.05M acetate buffer at pH 5.2. NFK-62-lysozyme was eluted in the peak underlined (OL-1).

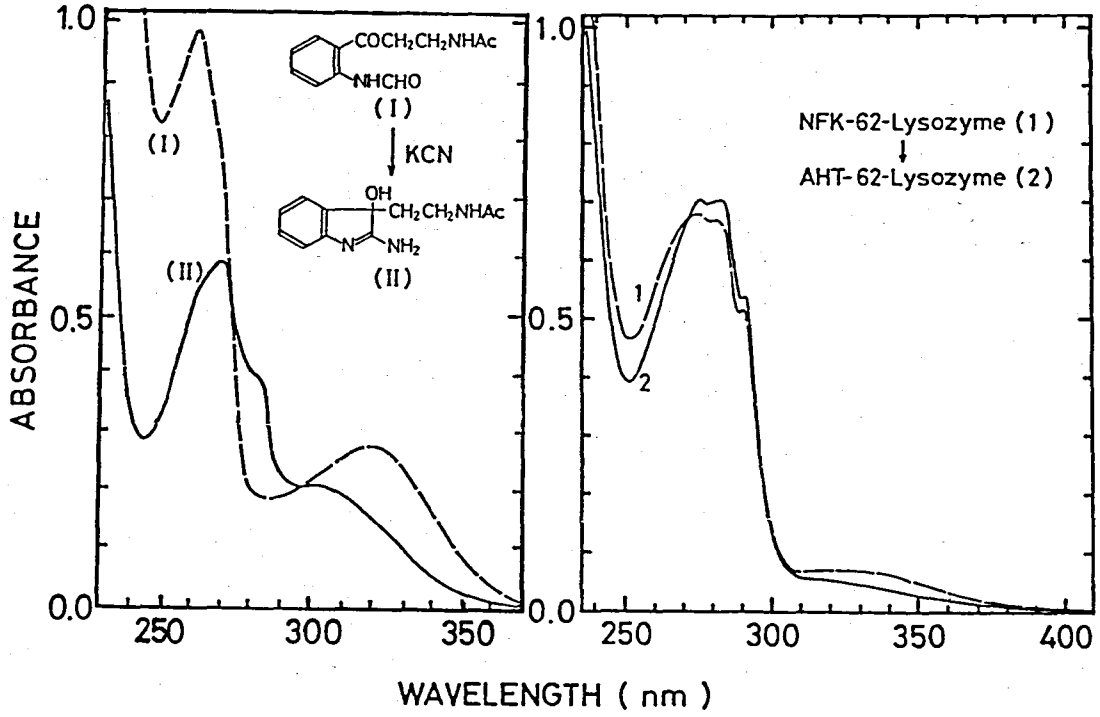


Fig. 2-2A. UV spectra of N-formylanthraniloyl compounds and 2-amino-3H-indol-3-ols. Right: NFK-62-lysozyme (1) and its reaction product with cyanide (2). Left: N-[2-(N-formylanthraniloyl)ethyl]acetamide (I) and N-[2-(2-amino-3-hydroxy-3H-indol-3-yl)ethyl]acetamide (II).

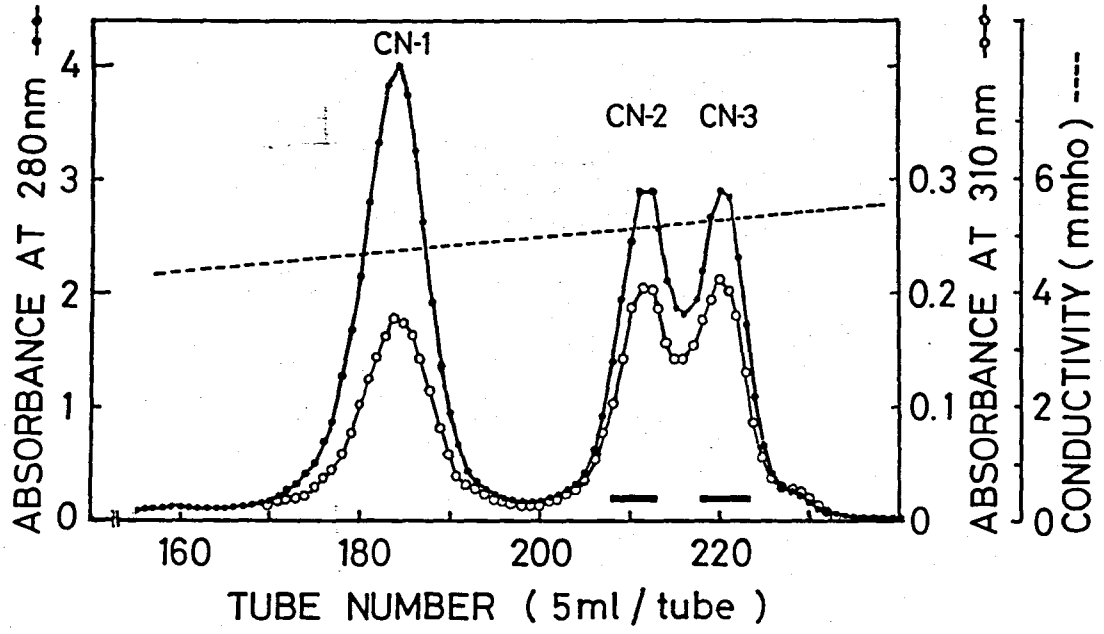


Fig. 2-2B. The ion-exchange chromatography of the reaction product of NFK-62-lysozyme with $K^{13}CN$. The reaction mixture was directly loaded on a column (2.2 x 120 cm) of CM-Sepharose CL-6B and eluted in the same manner as described in the legend of Fig. 2-1. AHT-62-lysozyme isomers were eluted separately in fractions CN-2 and CN-3.

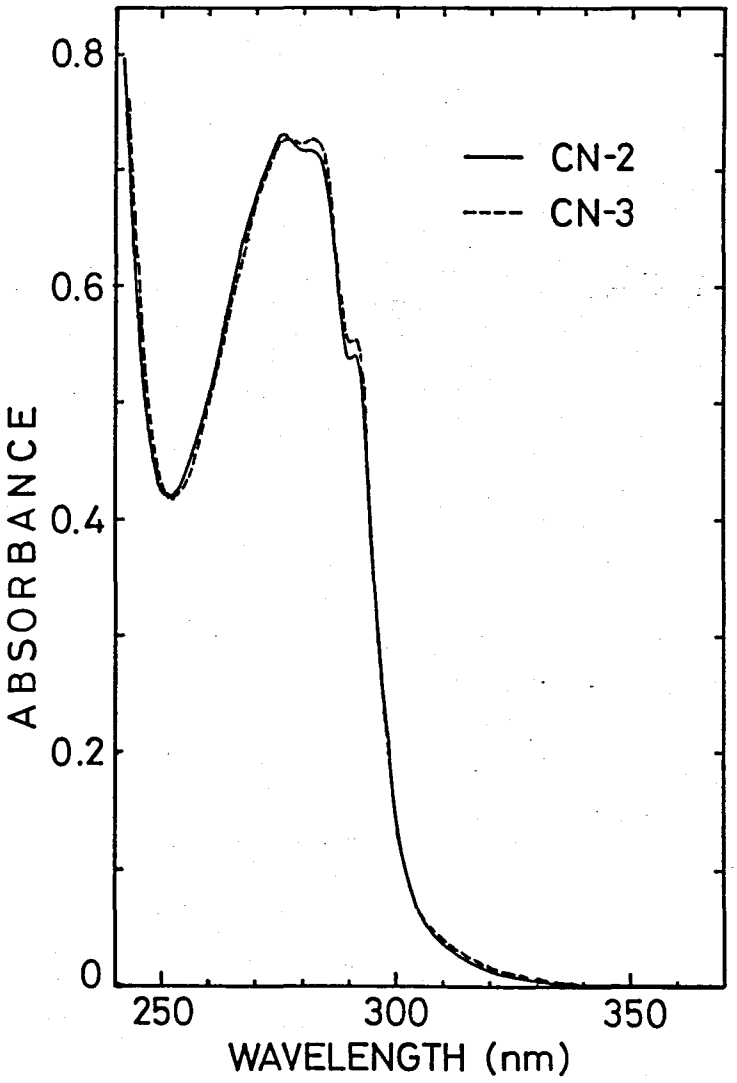


Fig. 2-3. UV spectra of AHT-62-lysozyme isomers.

Protein concentrations: ca. 1 mg/3.0 ml of 0.1M acetic acid.

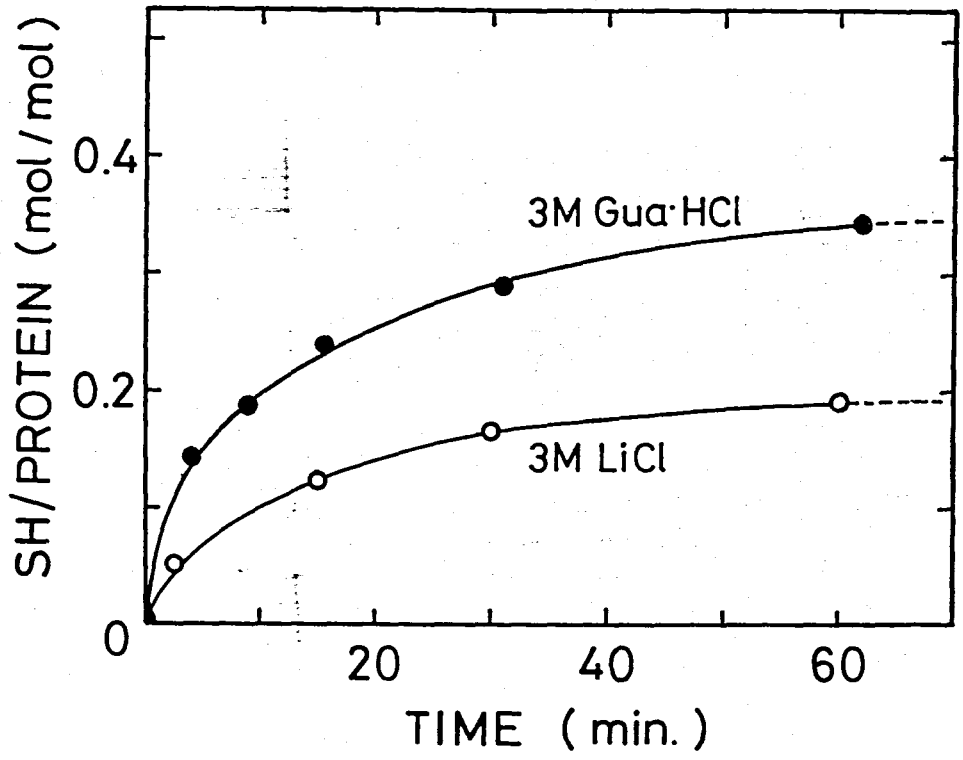


Fig. 2-4. Liberation of thiol groups during the reduction with $NaBH_4$. A protein sample (40 nmol) was withdrawn from the reaction mixture and reacted with 5,5'-dithiobis-(2-nitrobenzoic acid) in 3.0 ml solution at pH 8.0. The quantity of thiol groups liberated was calculated from the molar extinction coefficient of 13600 for 2-nitro-5-mercapto-benzoic acid at 412nm.

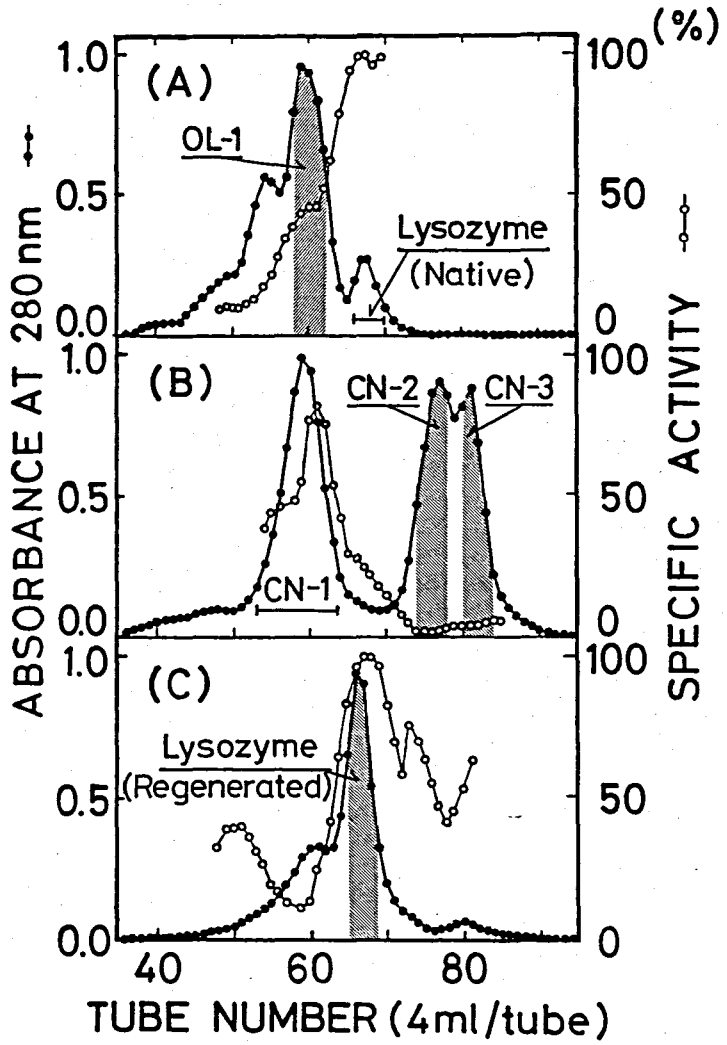


Fig. 2-5. Ion-exchange chromatography of lysozyme and its derivatives on CM-Sepharose CL-6B. (A) Crude NFK-62-lysozyme. (B) Reaction products of NFK-62-lysozyme (OL-1 in (A)) with CN^-. (C) The $NaBH_4$-reduction products of AHT-62-lysozyme (CN-3 in (B)) after acid-treatment. Chromatography was carried out with a linear concentration gradient of NaCl from 0.25 M (500 ml) to 0.75 M (500 ml) in 0.05 M sodium acetate buffer at pH 5.2 in the flow rate of 15 ml/hr. Quantities of samples loaded for (A)-(C) were 25-30 mg. Peak numbers, see text. Native lysozyme was eluted in tubes #65-70.

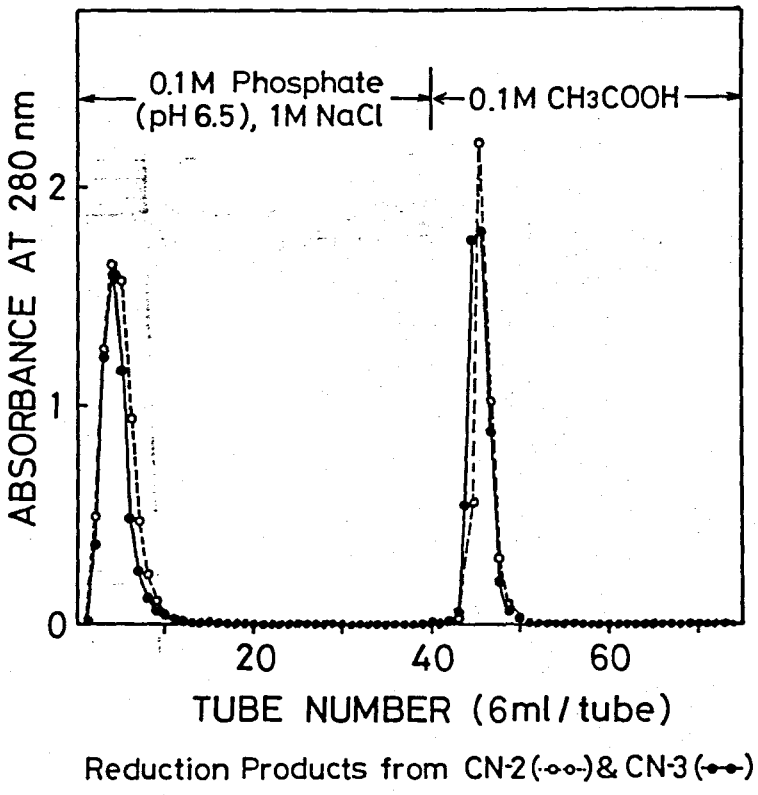


Fig. 2-6. Affinity chromatography of crude regenerated lysozyme on chitin-coated cellulose. The reaction mixture of protein CN-2 (or CN-3) with $NaBH_4$ was acidified, dialyzed, and diluted with an equal amount of 0.2 M phosphate buffer containing 2 M NaCl, pH 6.5, and loaded on a chitin-coated cellulose column (1.6 x 7 cm). Buffers for chromatography, see the Figure. Both experiments started from 30 mg of proteins CN-2 and CN-3.

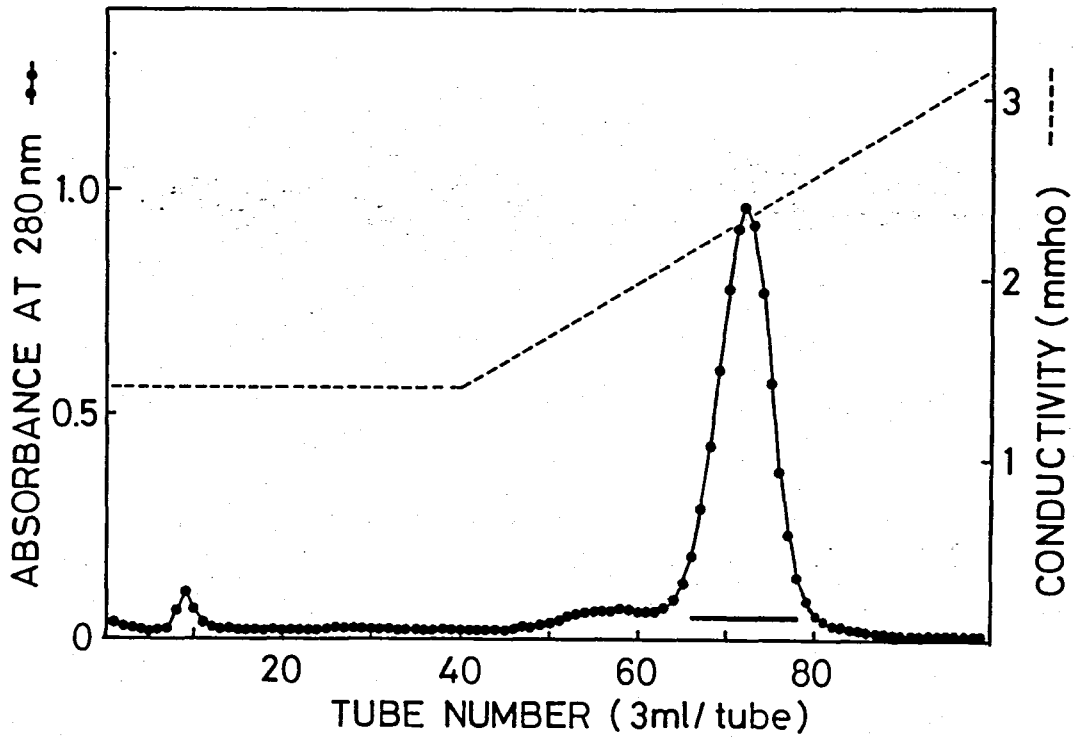


Fig. 2-7. Ion-exchange chromatography of crude regenerated lysozyme on Bio-Rex 70. Crude lysozyme (15 mg) adsorbed on chitin-coated cellulose was dissolved in 0.05 M sodium borate buffer (pH 10) and chromatographed on a Bio-Rex 70 column (1.2 x 43 cm) equilibrated with the same buffer. Elution was carried out with the borate buffer (120 ml) and then with a linear concentration gradient of NaCl from 0 (500 ml) to 0.8 M (500 ml) in the same buffer. The flow rate was 12 ml/hr.

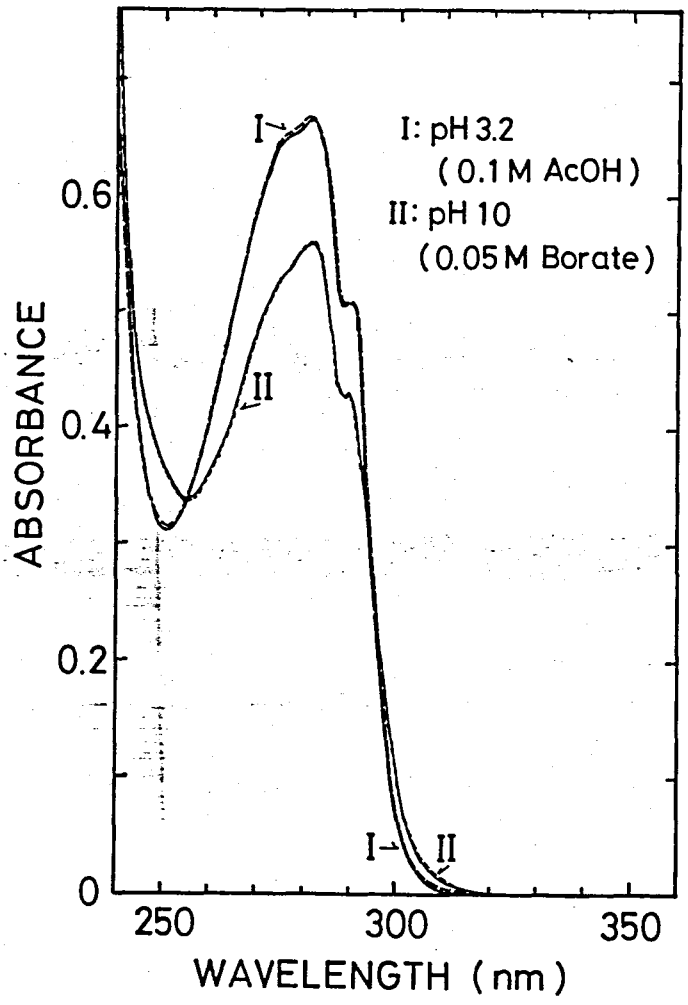


Fig. 2-8. UV spectra of native and regenerated lysozyme.

I : Recorded for the desalted ^{13}C-labeled protein after CM-Sepharose CL-6B chromatography.

II : Recorded for the protein in fraction #75 of Fig. 2-7.

Protein concentrations were arbitrary for spectra I and II. Native and regenerted lysozyme were dissolved in the same buffers shown in the Figure. Concentrations of both proteins were adjusted to the same absorbances at 280 nm each other.

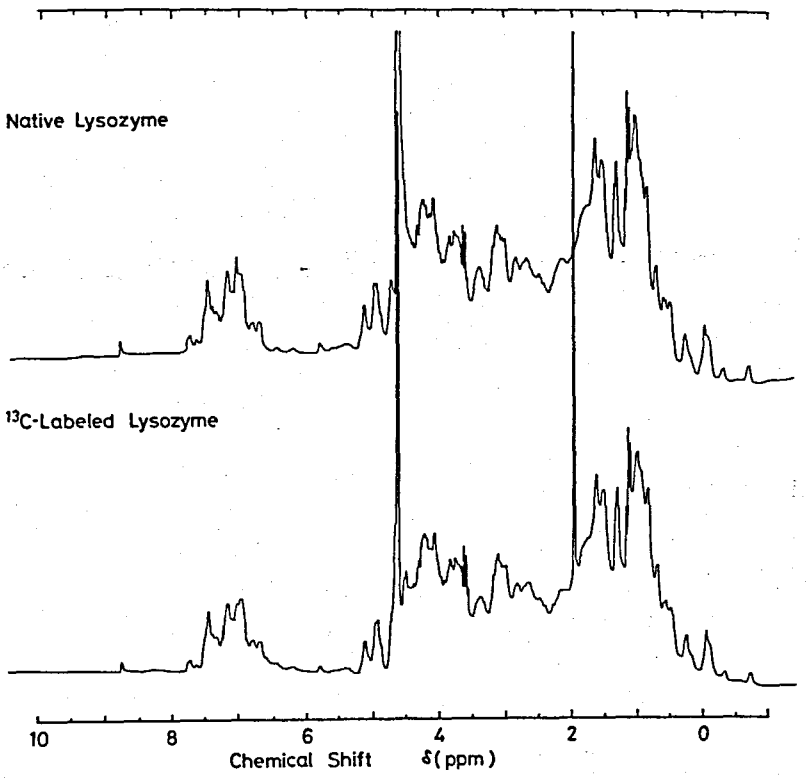


Fig. 2-9. 200 MHZ ^{1}H-NMR spectra of native and ^{13}C-labeled lysozyme. Spectra ewre recorded for 2 mM $^{2}H_2O$ solutions containing 0.1 M NaCl at p^{2}H 4.3 and 37°C after 8000 scans with a recycle time of 1.5 s. H^{2}HO signal was used as internal reference (4.64 ppm). A peak at 2 ppm is due to acetic acid contaminated during the preparation of ^{13}C-labeled lysozyme.

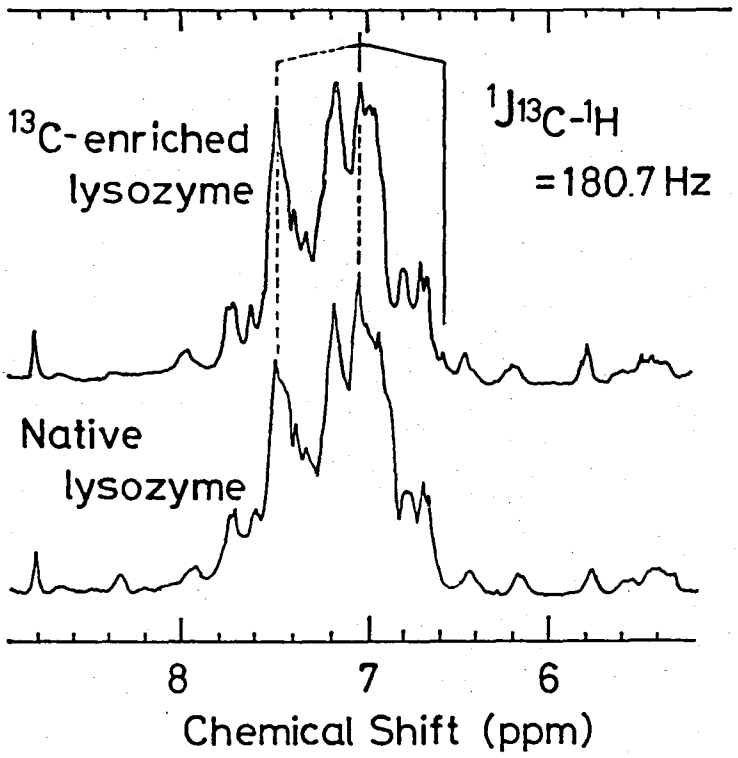


Fig. 2-10. 200MHz ^{1}H-NMR spectra of native and ^{13}C-enriched lysozyme in the region of aromatic protons. Upper ; ^{13}C enriched lysozyme, and lower ; native lysozyme. Spectra were obtained as shown in Fig. 2-9. The part of aromatic proton is expanded.

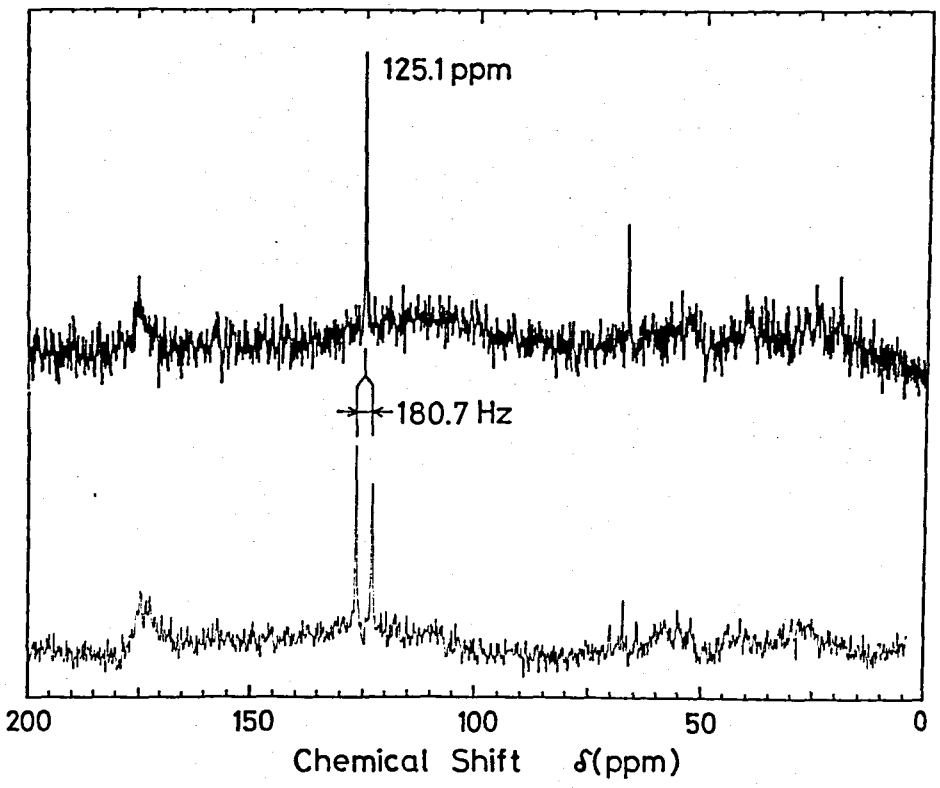
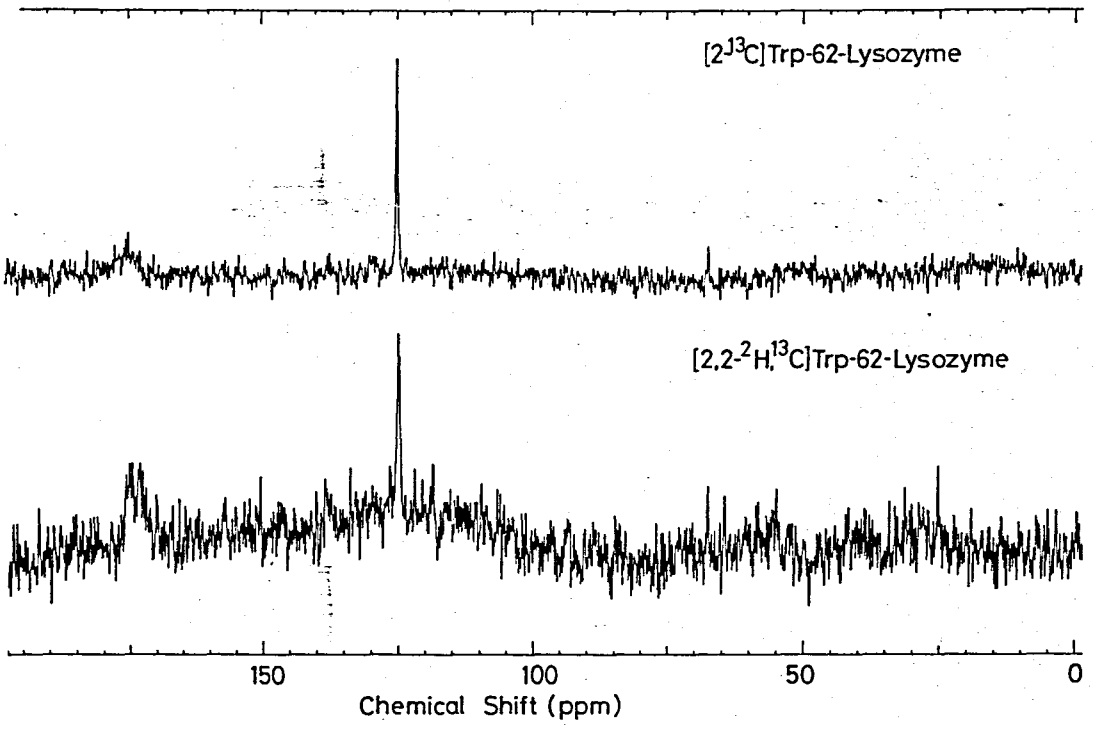


Fig. 2-11. 50.10MHz ^{13}C-NMR spectra of ^{13}C-enriched lysozyme in 0.5mM $^{2}H_2O$ solution containing 0.1M NaCl at p^{2}H 4.7 and 37°C.
Upper : Fully proton decoupled spectrum after 12000 scans with a recycle time of 0.6s.
Lower : Gated proton decoupled spectrum after 35000 scans with a recycle time of 1.4s. The peak at 67.4ppm is internal dioxane in each spectrum.



Fig, 2-12. 50.10MHz ^{13}C-NMR spectra of [2-^{13}C]Trp-62-lysozyme and [2,2-^{2}H,^{13}C]Trp-62-lysozyme.

Upper : Selectively proton decoupled spectrum of [2-^{13}C]Trp-62-lysozyme in 0.5mM $^{2}H_2O$ solution containing 0.1M NaCl at p^{2}H 4.7, and 37°C. The proton signal at 7.01 ppm in ^{1}H-NMR spectrum for the same sample was irradiated at 58.2741kHz. The spectrum was obtained after 36000 scans with a recycle time of 0.6s.

Lower : Non-decoupled spectrum of [2,2-^{2}H,^{13}C]Trp-62-lysozyme in 0.5mM $^{2}H_2O$ solution containing 0.1M NaCl at p^{2}H 4.9 and 37°C. The spectrum was obtained after 36000 scans with a recycle time of 0.8s.

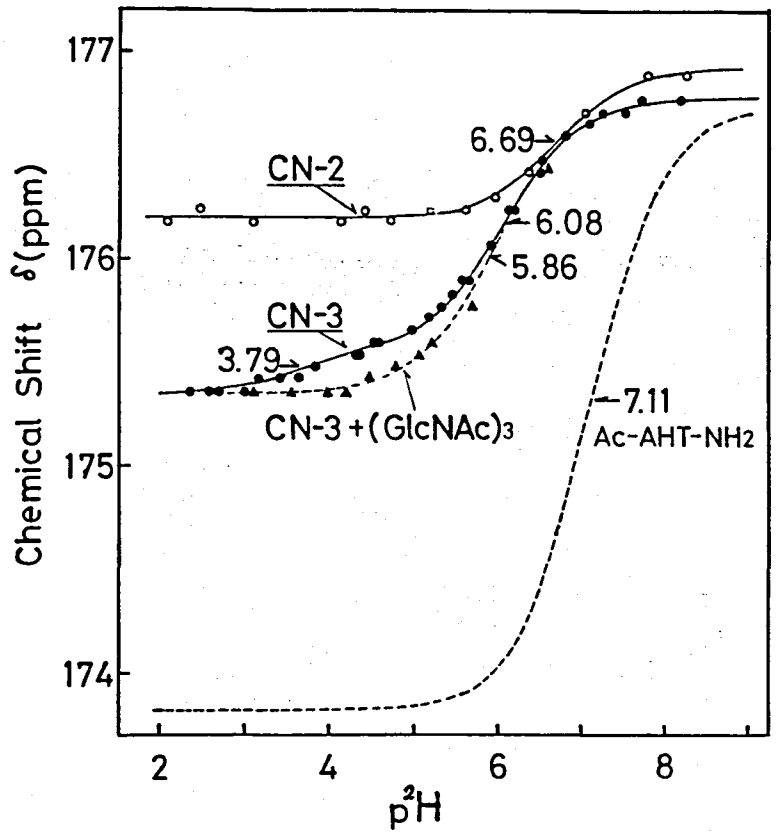


Fig. 2-13. ^{13}C-NMR titration curves of the 3H-indole C-2 carbon resonance of [2-^{13}C]AHT-62-lysozyme isomers (CN-2 and CN-3). For each data point, spectrum was accumulated approximately 8000 times with a recycle time of 1.5 s . The pKa values are presented in the figure and the deviations are ± 0.02 for CN-2 and CN-3, and ± 0.03 for CN-3 + $(GlcNAc)_3$.

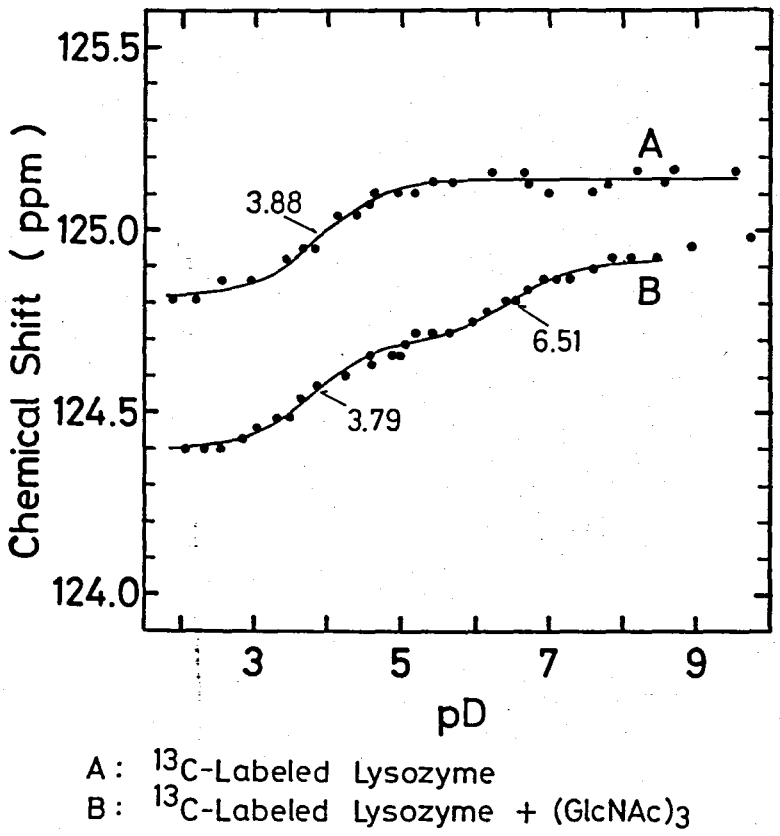


Fig. 2-14. ^{13}C-NMR titration curves for the enriched C-2 carbon resonance of [2-^{13}C]Trp-62-lysozyme in the presence (B) and absence (A) of chitotriose.

For each data point, spectrum was accumulated approximately 8000 times with a recycle time of 0.6s.

The pKa values are presented in the figure and the deviations are ± 0.02 for each value.

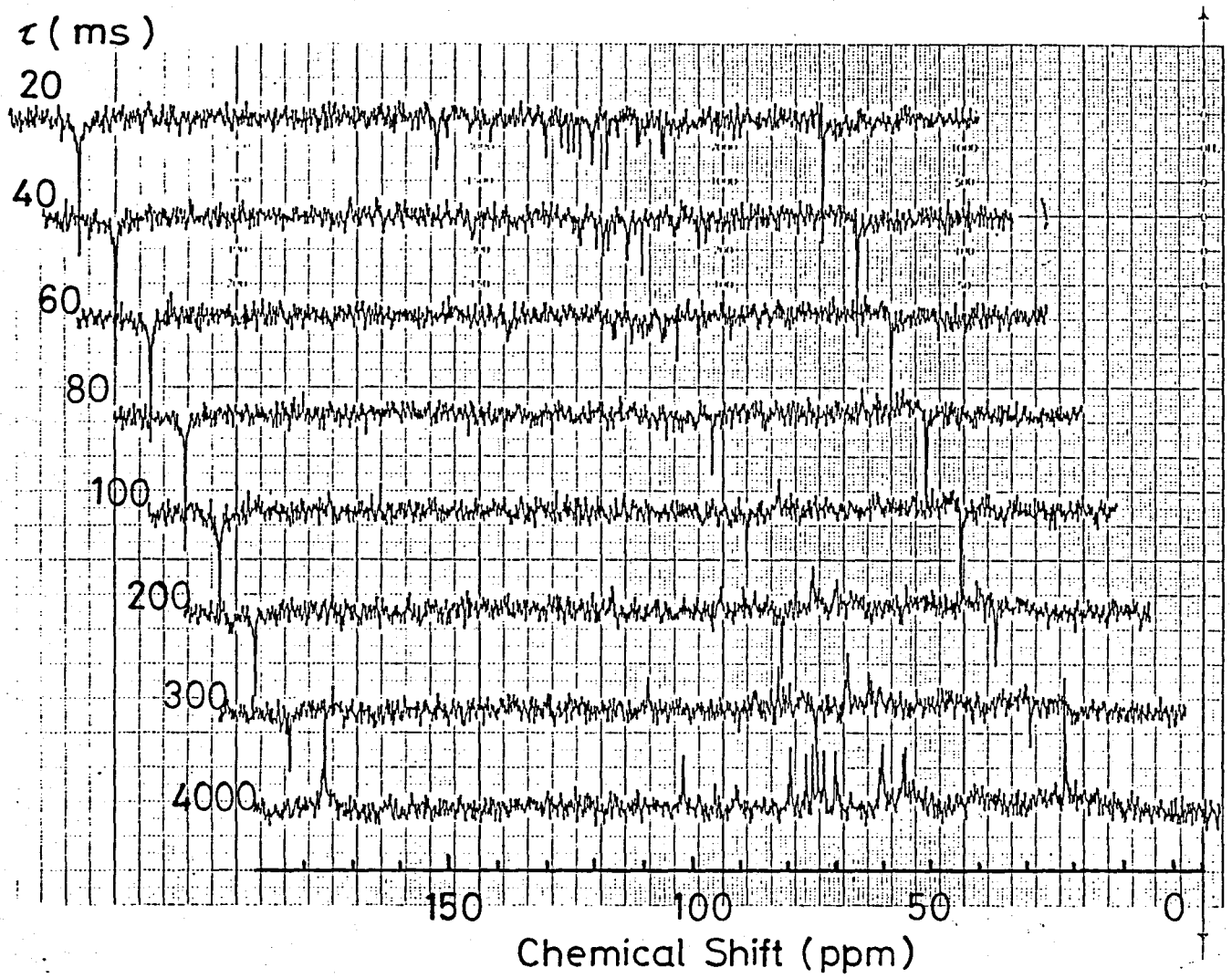


Fig. 2-15. Measurement of the spin-lattice relaxation time (T_1) for the enriched C-2 carbon of [2-^{13}C]AHT-62-lysozyme (CN-3) in the presence of a 10-fold excess of chitotriose at p^{2}H 4.75 and 37°C. Spectra were obtained after 4000 scans each. The C-2 carbon resonance of AHT appears at 175.48ppm.

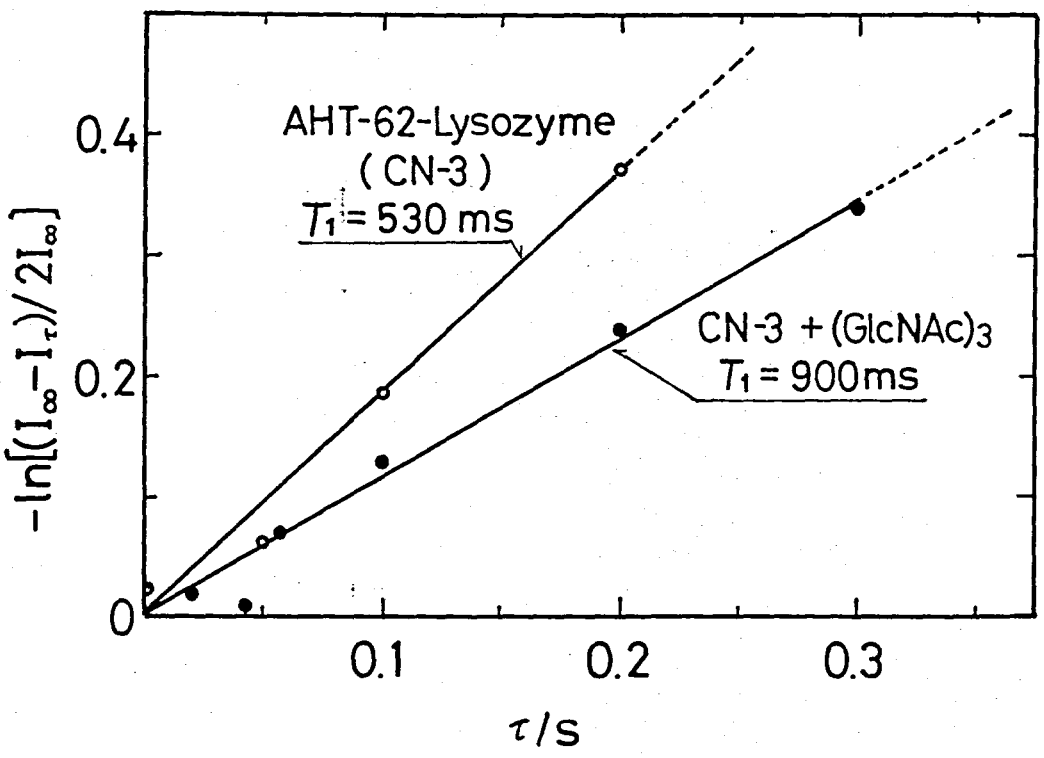


Fig. 2-16. Calculation of T_1 values for the C-2 carbon of AHT-62 in the presence and absence of chitotriose.

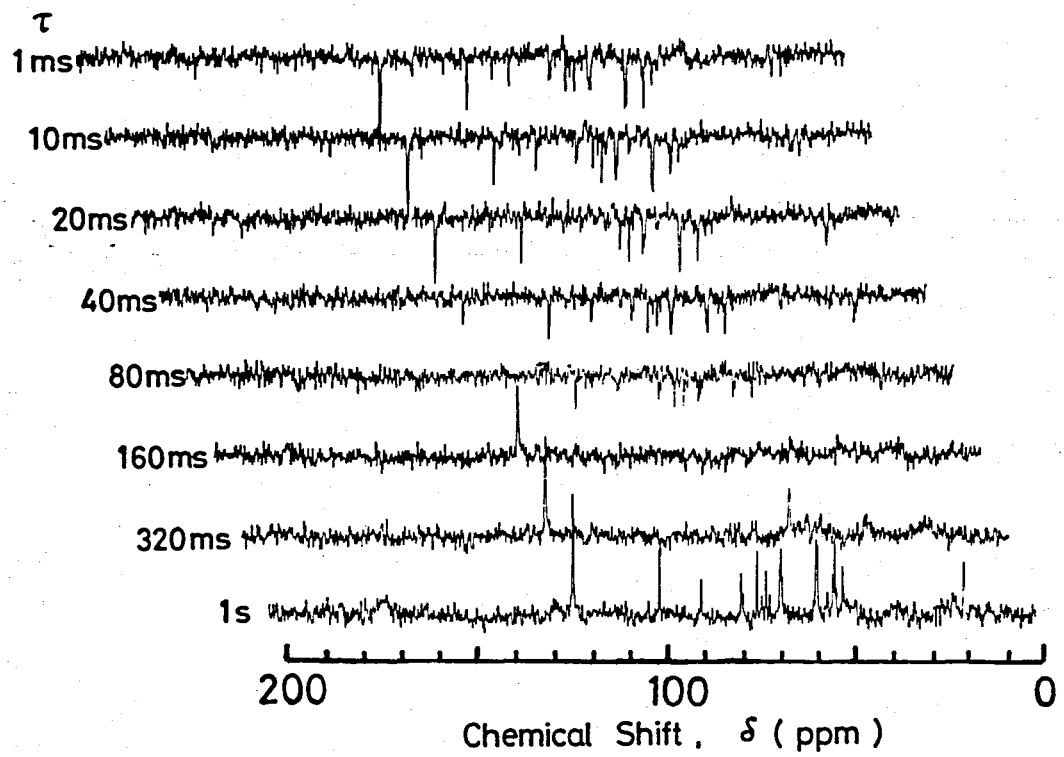


Fig. 2-17. Stacked spectra for the measurement of the T_1 value for C-2 carbon of Trp-62 in hen egg-white lysozyme in the presence of 10-fold molar excess of chitotriose at p^2H 4.9 at 37°C. Spectra were obtained after 10000 scans each. The C-2 carbon resonance appears at 124.66ppm.

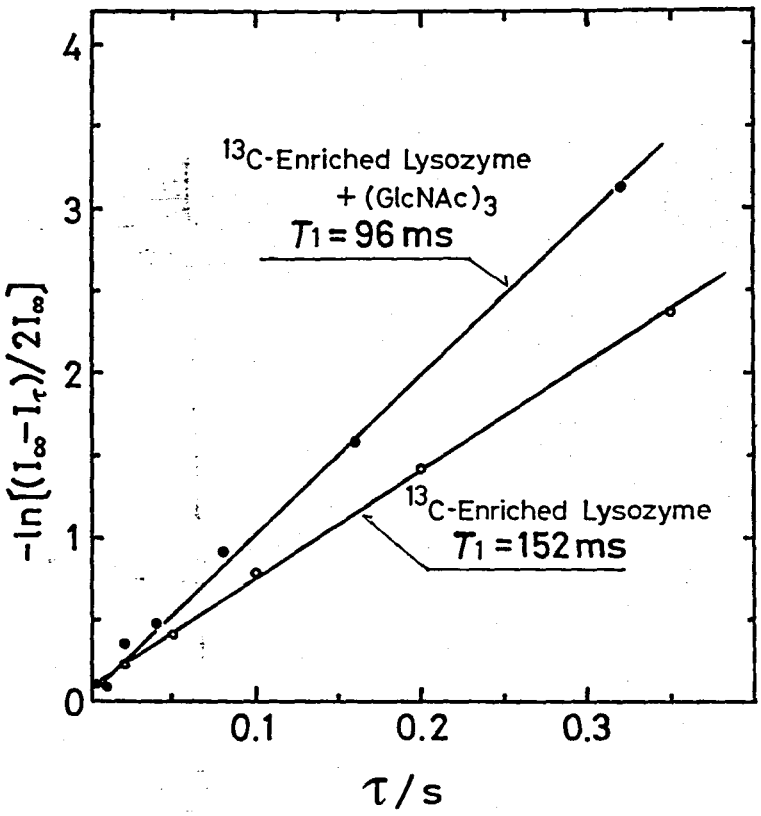


Fig. 2-18. Calculation of T_1 values for the C-2 carbon of Trp-62 in the presence and absence of chitotriose.

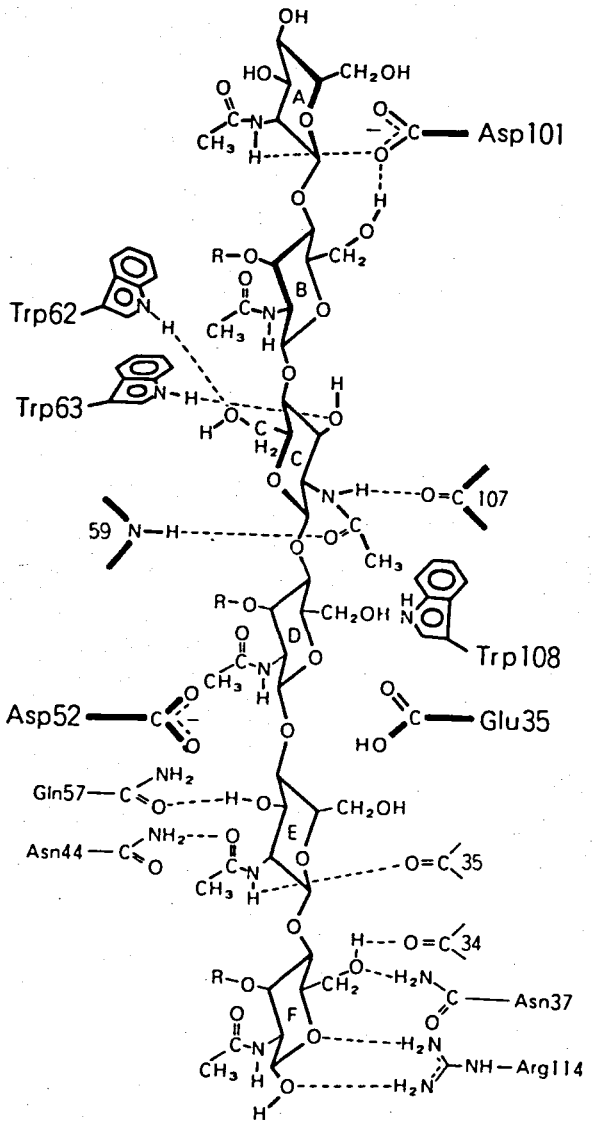


Fig. 2-19. Schematic illustration of the interaction of $(GlcNAc)_6$ with active-site residues of hen egg-white lysozyme. Reproduced from the figure presented by Hamaguchi and Kuramitsu (53).

SUMMARY

A new chemical method for the isotope labeling of indole C-2 atoms has been established. This method consists of the following reactions : ozonolysis of 3-alkylindoles to N-formylanthraniloyl compounds, cyclization of the latter to 2-amino-3-alkyl-3H-indol-3-ols by cyanide anion, and reduction of the 3H-indole with sodium borohydride to a 3-hydroxyindoline derivative which then reverted to the starting 3-alkylindole by the acid-catalyzed dehydration. The labeling with isotopes of carbon and hydrogen are achieved by the use of labeled cyanide and borohydride, respectively. For the application of this method to tryptophan residues in peptides and proteins, reaction conditions are further improved in several points. First, the cyclization of N-formylanthraniloyl compound to the 3H-indole was shown to be favourable even at 0°C in weakly basic aqueous media containing cyanide and a primary amine. The presence of the amine is important under the above conditions because the reaction essentially requires an acceptor of the formyl group. This finding led to the discovery of a new amide bond formation based on this acyl-transfer reaction. Second, 2-amino-3H-indol-3-ols are effectively reduced with sodium borohydride in a medium containing 3-4M LiCl (or NaCl, guanidium chloride) and 0.2% EDTA at initially a slightly acidic pH at room temperature. Such conditions are supposed to facilitate the ionic interaction of borohydride and the protonated 3H-indole.

According to these conditions thus established, hen egg-white lysozyme was labeled with ^{13}C at Trp-62 selectively. The labeled lysozyme retained full lytic activity, and was identical with native lysozyme in all respects tested. For this protein, the single ^{13}C nuclear magnetic resonance signal due to the enriched indole C-2 carbon of Trp-62 was detected at a reasonable position (125 ppm).

The pH dependences and the spin-lattice relaxation times (T_1) of this signal were studied in the presence and absence of chitotriose, a competitive inhibitor of lysozyme, to elucidate the role and the environment of Trp-62 in the catalytic function of lysozyme. For reference, diastereoisomers of [2-^{13}C]-aminohydroxytryptophan-62-lysozyme, the intermdiary products obtained during the preparation of [2-^{13}C]Trp-62-lysozyme, were studied as above.

Consequently, it was revealed that the catalytic groups, Glu-35 and Asp-52, principally influenced the conformation change at or around Trp-62 depending on their dissociation equilibrium. The T_1 measurements suggested the importance of the flexibility of Trp-62 for the substrate binding of lysozyme.

These results as well as the success in the labeling of Trp-62 in lysozyme indicated the availability of this method for the labeling studies of tryptophan residues in other proteins.

ACKNOWLEDGEMENTS

The author is grateful to the late Professor Kozo Narita for the guidance and encouragement. The author is also indebted to Assistant Professor Fumio Sakiyama for his helpful advice and discussions throughout this work. The author thanks Professor Yoshimasa Kyogoku, Drs. Yuji Kobayashi and Hideo Akutsu and the members of Division of Molecular Biophysics, Institute for Protein Research, Osaka University, for NMR measurements, Dr. Yasuo Hata of this institute for X-ray crystallographic analysis, and Miss Yoshiko Yagi of this institute for amino acid analyses. The author's thanks are also due to Assistant Professor Nobuyuki Yamasaki, Kyushu University, and Assistant Professor Masatsune Kainosho, Tokyo Metropolitan University for their kind advices. The author is also grateful to Dr. Nobuhito Masuda, Fuji Photo-film Co. Ltd., Dr. Yasutsugu Fukunaga, Georgia Institute of Technology, Mr. Kensaku Mizuno, Miyazaki Medical College, and Mr. Takashi Izumi for their stimulating discussions.

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