ACTA CHEMICA SCANDINAVICA 23 (1969) 3051–3054

Isotopic Labelling of Tryptophan and Tryptophan Residues in Polypeptides

BØRGE BAK, JENS JØRGEN LED and ERIK JONAS PEDERSEN

Chemical Laboratory V, University of Copenhagen, Copenhagen, Denmark

In a previous communication [1] hydrogen-deuterium exchange was shown to occur at positions 1,2,4,5,6, and 7 (the indolyl moiety) in tryptophan dissolved in anhydrous CF_3COOD. None of the "aromatic" amino acids phenylalanine, tyrosine, and histidine and their corresponding amino acid residues in polypeptides such as insulin and glucagon show this reaction. This paper gives a practical procedure for carrying out the selective deuteration and tritiation of the single tryptophan residue in glucagon. The method seems capable of working for most polypeptides and proteins. In reverse, selective *protonation* of biologically prepared perdeuterio polypeptides should be feasible.

EXPERIMENTS WITH TRYPTOPHAN

50 mg (0.24 mmole) of tryptophan (I) was shaken for 1 h at room temperature with 1 ml D_2O for removal of readily exchangeable protons bound to N and O. Water was distilled off in a vacuum and replaced by 1000 μl (13.5 mmoles) of CF_3COOD (II) containing 98.0 ± 1.0 % D. In (I) only the α- and the β-protons remain unexchanged so that (I) has 11 exchangeable protons when dissolved in (II). The maximum obtainable mole ratio of (I)-d_{11} is $0.98^{11} = 0.80 \pm 0.10$. The maximum obtainable mole ratio of 2,4,5,6,7(I)-d_5 (not specifying if H or D occupy the sites NH_2^+, NH_3^+, and COOH) is $0.98^5 = 0.90 \pm 0.05$.

^{1}HNMR (100 MHz) spectra of (I) in (II) after 1, 2, 5, and 6 h at 42° showed that exchange had essentially stopped after 5 h as judged by the ratio of the integrated area under the linegroup of still unexchanged protons in positions 1(NH_2^+), 2, 4, 5, 6, 7, and NH_3^+ (10 protons) to the area under the signals from the unexchangeable α- and β-protons (3 protons). After 5 and 6 h this ratio was 1:3 meaning that 1 out of 10 exchangeable protons was still present. Therefore, the mole ratio of (I)-d_{11} is $0.90^{11} = 0.314$ at this stage. The solvent was removed *in vacuo*, replaced by 1000 μl of (II) *etc.* as above. The resulting mole ratio of (I)-d_{11} was 0.715. After a third treatment with 1000 μl of (II) the mole ratio of (I)-d_{11} was 0.828 (as seen by accumulating 25 spectra) corre-

sponding to a mole ratio of 2,4,5,6,7(I)-d_5 of 0.918, or maximum obtainable deuteration by 98 % CF_3COOD in the 4 operations indicated. We shall refer to the final solution as (T).

(T) is chemically very stable. Part of it was left for several weeks at room temperature. Its ^{1}NMR spectrum was unchanged (α- and β-protons). After evaporation and addition of CF_3COOH *etc.* it was reconverted quantitatively to (I) as seen by its ^{1}HMR spectrum.

10.5 mg (0.164 mmole) of CD_3COOD was added to 500 μl of (T) to act as an internal standard for frequency and intensity by its CD_3 signal. The ^{2}HMR (15.4 MHz) spectrum of the solution was recorded. A 15 cps broad signal situated 4.55—4.86 ppm downfield from the CD_3 resonance was observed. 5.0 ± 0.5 deuterium resonances per mole of (I)-d_{11} species were found by area integration. The deuterons in ND_2^+, ND_3^+, and COOD exchange so rapidly with the deuteron of (II) that they are unable to produce separate signals at 15.4 MHz although they do so at 100 MHz. Replacement of (II) by 0.1 N HCl produced a ^{2}HMR spectrum showing a broad, but permanent ^{2}HMR signal 4.55—4.86 ppm downfield from internal CD_3COOH corresponding to 4 ± 1 deuterium resonances per molecule of deuterated (I). When the ^{1}HMR spectrum of this sample was recorded, no signals from aromatic protons were observable. The inference that a polypeptide tryptophan residue deuterated in its "indolyl" moiety will not exchange D by H in acid aqueous medium is not justified.

50 μl of (T) containing 0.012 mmole of deuterated (I) was diluted by 550 μl of (II) containing 0.016 mmole CD_3COOD. The ^{2}HMR spectrum was recorded in order to test how to find and to estimate the intensity of a ^{2}HMR signal when working in approximately the deuterium concentration which will occur in a 10 % solution of glucagon (III), deuterated in its indolyl moiety of its single tryptophan residue. *Ca.* 200 accumulated spectra proved suitable. A broad signal was observed at the expected position. Dependent upon the number of accumulated spectra, the choice of spectral baseline and the intensity of the "analytical" electromagnetic field of the instrument, the estimated intensity of the ^{2}HMR signal varied between 3.4 and 4.0 per mole of deuterated (I). The apparent loss of signal corresponding to 1—2 deuterium resonances demonstrates the difficulty in working with weak, broad lines.

EXPERIMENTS WITH GLUCAGON

50 mg (0.0143 mmole) of glucagon (III) was shaken for 24 h at room temperature with 5 ml D_2O. Water was distilled off in a vacuum at room temperature. The remaining (III), in which practically all N- and O-bond H had been replaced by D, was dissolved in 500 μl (6.75 mmoles) CF_3COOD (98 %). After 1 h at 30°, 25 ^{1}HMR (100 MHz) spectra were accumulated over a period of 45 min (spec. 1) and this was immediately repeated (spec. 2). The solvent was removed *in vacuo* and replaced by 500 μl of (II). Again, 25 ^{1}HMR spectra were recorded twice as above (spec. 3 and 4). We shall refer to the resulting solution as (G). The ^{1}HMR spectrum of glucagon has a well-defined band[2] between 3.90 and 6.00 ppm/TMS representing 41 proton resonances (p.r.). This band was used as an internal intensity standard to estimate the number

of p.r. in the region 6.40—8.00 ppm/TMS which, for (III) with an unaffected tryptophan residue would be 24, namely, 10 phe_{arom} +8 tyr_{arom} +1 his(4) +5 try(2,4,5,6,7). Spec. 1—4 showed the presence of 19.7, 19.3, 18.9, and 18.7 p.r., respectively, satisfactorily approaching the expected 19.0 p.r. No corresponding intensity loss occurs for, *e.g.*, insulin containing 3 phenylalanine, 4 tyrosine, and 2 histidine, but no tryptophan residues.[2,3]

Next, it was attempted to find a ^{2}HMR signal from the deuterated glucagon of proper position and intensity. 3—400 spectra were accumulated. A broad signal at the expected position 4.55—4.86 ppm downfield from internal CD_3COOD was recorded. Its integrated intensity corresponds to *ca.* 3 d.r. per mole of deuterated (III). Obviously, deuterium resonances from the numerous N—D and O—D groups coincide with the CF_3COOD resonance. Within the limits of error this result is in harmony with evidence from the ^{1}HMR spectra above in view of what was found for highly dilute (T).

TRITIATED, BIOLOGICALLY ACTIVE GLUCAGON

When (III) is dissolved in (II) or in CF_3COOH its 7 OH groups of the 4 serine and the 3 threonine residues slowly convert to $OCOCF_3$ as seen by observation of ^{19}FMR spectra.[4] The ester groups may be hydrolyzed by shaking solid heptatrifluoroacetylated (III) with water for 24 h at room temperature. ^{1}HMR and ^{19}FMR spectra show this.[4] The glucagon recovered has the expected biological activity.[4] Therefore, in order to remove the $COCF_3$ groups from (III) in (G), the solvent was evaporated and replaced by 1000 μl H_2O and 10 μl CF_3COOH. After shaking for 24 h and evaporation to dryness the residue was dissolved in 0.1 N HCl (500 μl) since it would now be meaningless to apply (II) or CF_3COOH. However, the solution was viscous and perhaps colloidal. No ^{2}HMR signals were observed. This could be due, either to unfavourable spectroscopic conditions or to deuterium-hydrogen exchange during the hydrolysis. In a following experiment tritiation was applied. 49.8 mg (0.0143 mmole) of (III) was dissolved in 1500 μl of tritiated CF_3COOH* made from 1500 μl $(CF_3CO)_2O$ (10.7 mmoles) and 200 μl (11.1 mmoles) tritiated water (specific activity 10.0 μC). After 2 days the solvent was distilled off and tested for radioactivity applying conventional liquid scintillation techniques with corrections for "quenching" and "background" effects. 100 μl of the distillate produced 76 000 $\pm$ 2000 cpm. Therefore, 1 mmole of H* produces 57 800 cpm. From the spectroscopic experiments it follows that H* of CF_3COOH* is in exchange equilibrium (after 2 days) with 5 tryptophan residue protons (2,4,5,6,7) and 61 other glucagon protons in —CONH—, $=NH_2^+$, $-NH_2^+$, $-NH_3^+$, $-CONH_2$, —COOH, and —OH groups. Consequently, 0.0143 mmole of heptatrifluoroacetylated glucagon must produce 4133 cpm from its tryptophan residue and 50 420 cpm from the rest of the molecule. Now, to produce biologically active glucagon, the 7 $COCF_3$ groups were removed by hydrolysis as described above (24 h contact with water). This treatment also removed the major part of the radioactivity associated with the non-tryptophan residue protons. For completion, glucagon was shaken with 1 ml water for 16 h. The water was distilled off and the operation was repeated. The second distillate showed only twice the "background" radio-

activity. The weight of the remaining salt of glucagon ($+6CF_3COOH$, formula weight 4166) was 57.9 mg or 0.0139 mmole. 6 mg of the salt was dissolved in 500 μl water and the radioactivity of the solution was measured. 119 ± 2 cpm were found, or 1148 cpm for the total glucagon quantity. During hydrolysis *etc.* this glucagon had been in contact with water for 56 h. Without exchange of the tryptophan residue protons (2,4,5,6,7) 4133 cpm (above) would be expected. We conclude that such exchange has occurred, although slowly, reducing the radioactivity associated with the indolyl part of the tryptophan residue of glucagon to *ca.* 30 % of its value prior to hydrolysis of heptatrifluoroacetylated glucagon. Precipitation of glucagon by 10 % (vol./vol.) CCl_3COOH in water showed that only 10 % of the radioactivity remained in the supernatant liquid.

Acknowledgements. We are indebted to Dr. Jørgen Schlichtkrull, the NOVO Research Institute, Copenhagen for gifts of highly purified glucagon, and to Klaus Jørgensen of the INSTITUTE for measuring the activity of our radioactive samples.

Note added in proof. Recent experiments [4] at 220 MHz and 25° show that exchange of aromatic tryptophan protons in glucagon dissolved in $CF_3COOD(CF_3COOH)$ should be interrupted after 4 h, not 2 days as above. It is unknown if this will raise the biological activity of retrieved glucagon.

REFERENCES

1. Bak, B., Dambmann, C. and Nicolaisen, F. *Acta Chem. Scand.* **21** (1967) 1674.
2. Bak, B., Pedersen, E. J. and Sundby, F. *J. Biol. Chem.* **242** (1967) 2637.
3. Bak, B., Bhacca, N. S., Dambmann, C., Nicolaisen, F. and Pedersen, E. J. *J. Mol. Spectry.* **26** (1968) 78.
4. Bak, B., Led, J. J. and Pedersen, E. J. *Kgl. Danske Vidensk. Selskab, Mat.-Fys. Medd.* **37** (1969) 8.

Received March 22, 1969.