SELECTIVE SUBSTITUTION OF ^2H AND ^3H INTO AROMATIC AMINO ACIDS CATALYZED BY RANEY NICKEL

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Received 22 February 1979

1. Introduction

Proton magnetic resonance studies of complex molecules, such as peptides and proteins, are often severely limited by problems of resolution and assignment of specific resonances in the spectrum [1]. Considerable simplification of such problems can result from the substitution of ^2H or ^3H for specific ^1H atoms in the molecule [2,3]. Provided that the positions of substitution are known, not only are the number of spectral resonances reduced, but the resonances which remain, or are missing, may be assigned. In addition, the spin–spin coupling and relaxation behaviour of the remaining protons may be considerably simplified. The application of ^2H or ^3H magnetic resonance to the substituted molecule may also be of value.

Two approaches to the substitution of hydrogen are possible. Either exchange of ^2H or ^3H for specific hydrogens in the intact molecule must be effected, or the molecule must be synthesized from specifically deuteriated or tritiated precursors. Both approaches have been used successfully in prior studies of peptides and proteins. In intact proteins, the hydrogens of amide nitrogens can exchange with those of the solvent, and replacement by deuterium considerably simplifies the proton NMR spectrum. Of the CH hydrogens, those at the C(2) position in histidine can sometimes be conveniently exchanged by simple equilibration with ^2H_2O. In ribonuclease, specific assignments were possible using this method [4,5]. However, replacement of other CH hydrogens is rarely possible without marked chemical alteration of the molecule.

Synthesis of totally deuteriated proteins has been achieved by growing microorganisms in fully deuteriated media [6], and synthesis of proteins having selective deuteriation has been achieved by growing bacteria on a defined medium containing selectively deuteriated amino acids. This latter approach has achieved notable success in the studies of dihydrofolate reductase from *Lactobacillus casei* [7].

It is possible to replace directly certain of the CH hydrogens of certain amino acids, in particular the aromatic amino acids, by ^2H. Equilibrium of individual aromatic amino acids in ^2H_2O containing up to 85% $^2H_2SO_4$ or 75% trifluoro-[^2H]acetic acid at elevated temperatures or for extended times effects exchange [8,9] and has been used to prepare large amounts of deuteriated amino acids. However, frequently racemization at the α-carbon occurs, and the methods cannot be extended to the deuteriation of peptides or proteins because hydrolysis of the molecule occurs. Here we report the use of a catalytic method to permit selective exchange to be carried out under mild conditions. The catalyst employed is Raney nickel, which has been used to produce deuteriated phenols from selectively halogenated phenols [10] and has been reported to catalyze the exchange of aromatic protons for deuterons in phenylalanine to various degrees [11]. We report the preparation of selectively deuteriated derivatives of phenylalanine, tyrosine and tryptophan, and suggest the feasibility of extending the method to peptides and proteins.

2. Materials and methods

Amino acids were chromatographically pure prepa-

rations from the Sigma Chemical Co. or from Calbiochem. *N*-Acetyl-tyrosine amide was obtained from Mann Research Labs.

Raney nickel W-2 was prepared as in [12] but the heating step was limited to 1 h or 2 h at 50°C. The Raney nickel was placed in a Soxhlet extractor and washed free of excess alkali by pumping 200 vol. of glass distilled water over the catalyst within about 24 h. The catalyst was freed of H_2O by washing twice with 95% ethanol and twice with 2,4-dioxan, then placed in 2 vol. 99.8% ^2H_2O. The suspension was agitated on a rocking table in a closed tube for 2 days at room temperature, then for 2 more days with fresh ^2H_2O.

In a typical equilibration experiment 0.5 g (3.0 mmol) phenylalanine was dissolved in 5.0 ml 99.8% ^2H_2O and 0.3 ml 10 N NaO^2H (3.0 mmol). The solution was adjusted to pH 8–9 with ^2HCl or NaO^2H. Deuterized Raney nickel (~1 g) was added and the suspension in a screwcap centrifuge tube was agitated on the rocking table at room temperature. At various times the catalyst was centrifuged to the bottom of the tube and an aliquot of the supernate was removed for analysis. The catalyst was resuspended and agitation of the reaction sample continued. In a similar manner exchange reaction experiments were done with tryptophan, tyrosine and *N*-acetyl-tyrosine amide. Samples were freed of Ni^{2+} by passage through a column of Chelex-100 (Bio-Rad Labs). The column effluent was monitored at an appropriate wavelength in the ultraviolet so that the fractions containing amino acid could be pooled. Alternatively Ni^{2+} was precipitated as NiS.

Exchange of hydrogen for ^2H was followed as a function of time by analysis of the 270 MHz proton NMR spectra of the various samples after lyophilization and dissolution in ^2H_2O.

3. Results

In fig.1 are shown the aromatic proton resonances of tyrosine, phenylalanine and tryptophan before and after exposure to the catalytic exchange medium. For tyrosine and phenylalanine, even after extended equilibration, no significant exchange of the hydrogens *ortho* to the methylene group (C(2)H, C(6)H) occurred under the conditions used here. However, essentially

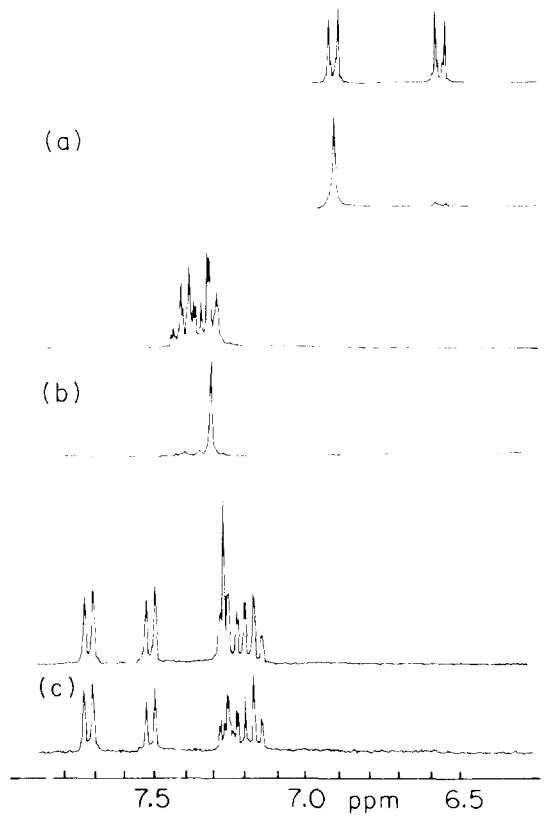

Fig.1. 270 MHz ^1H NMR spectra of (a) tyrosine, (b) phenylalanine and (c) tryptophan, before and after equilibration in the catalytic exchange medium for 114 h, 142 h and 48 h, respectively. The spectra in (a) are of solutions at pH 11. The others are at pH 9. The catalyst preparation of (b) was a different one from that of (a) and (c).

total exchange of the remaining aromatic hydrogens for deuterons occurred, resulting in spectra containing only a singlet aromatic proton resonance in both cases. In table 1 the relative rates of exchange for the different protons are given. It was found, however, that these rates depend on the exact nature of the catalyst preparation. In experiments with tryptophan, no significant exchange of the C(4)H, C(5)H or C(6)H hydrogens was observed. The C(2)H hydrogen exchanges considerably more rapidly than the C(7)H hydrogen, permitting preparations of tryptophan both singly and doubly deuteriated in the aromatic ring (see fig.1).

For all three amino acids, exchange of the aliphatic

Table 1
Kinetics of hydrogen exchange[a]

Amino acid	Hydrogen[b]	$t_{1/2}$ (h)	Relative exchange rate
Tyrosine	C(4)H, C(5)H	12	1.00
	C(2)H, C(6)H	–[c]	0.00
Phenylalanine	C(4)H, C(5)H	100[d]	0.12
	C(3)H	100[d]	0.12
	C(2)H, C(6)H	–[c]	0.00
Tryptophan	C(2)H	17	0.17
	C(4)H	135	0.09
	C(5)H	–[c]	0.00
	C(6)H	–[c]	0.00
	C(7)H	–[c]	0.00

[a] Measured for single catalyst preparation. pH 9.0, 20°C
[b] Nomenclature according to IUPAC recommendations (Eur. J. Biochem. 53, 1 (1978)). For both tyrosine and phenylalanine, C(2)H and C(6)H are *ortho* to the α-CH_2 group
[c] $t_{1/2} > 500$ h. No measurable exchange was observed at < 114 h
[d] Distinction between C(3)H and C(4)H, C(5)H resonances was not made

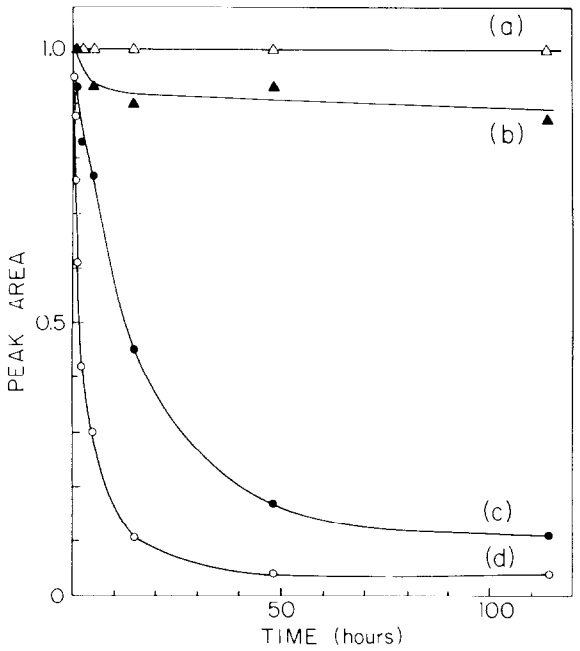

Fig.2. The fraction of hydrogen rather than ^2H at specific positions in the molecule of tyrosine, as a function of the time of equilibration in the exchange medium. The data are derived from peak areas in NMR spectra, measured relative to the area of the C(2)H, C(6)H, peak. That no exchange of the C(2)H, C(6)H hydrogens occurred was determined most readily by observing that the residual C(3)H, C(5)H peak was in all cases a doublet, with no singlet component. (a) C(2)H, C(6)H; (b) α-CH; (c) C(3)H, C(5)H; (d) β-CH_2.

sidechain hydrogens also occurred. Figure 2 shows a graph of normalized peak area against time for an experiment designed to follow the exchange of different hydrogens of tyrosine. The β-CH_2 hydrogens exchange rapidly ($t_{1/2} = 2.0$ h, rate relative to C(4)H, C(5)H = 6.0, whilst the α-CH hydrogen exchanges only slowly ($t_{1/2} > 500$ h, relative rate < 0.01). The exchange of the α-CH hydrogen was accompanied by a change in specific optimal rotation, indicating that this exchange results in racemization. However, the overall optimal activity of the deuteriated species remains high. When *N*-acetyl-tyrosine amide was subjected to exchange under the conditions used for the amino acids, full exchange of the C(2)H, C(6)H hydrogens, and of the β-CH_2 hydrogens was achieved, without measurable exchange of the α-CH, C(4)H, C(5)H or methyl group hydrogens. The NMR spectrum showed that significant hydrolysis of the molecule had not occurred.

4. Discussion

Here we have shown that selectively deuteriated aromatic amino acids can readily be prepared under mild conditions by using a catalytic method. We have also demonstrated that ^3H incorporation is possible by this method. The mechanisms and steric requirements for heavy metal catalysis of exchange of hydrogen for ^2H in substituted aromatic hydrocarbons have been studied extensively [13–16]. Our observation that hydrogen atoms *meta* and *para* to the alkyl side chains exchange readily in tyrosine and phenylalanine whilst those *ortho* to the side chain do not is consistent with studies of alkylbenzenes where nickel films were used as catalysts [13]. Our observation that the relative rate of exchange of different hydrogens in the amino acids was sensitive to the catalyst preparation is also consistent with this work [13].

As fig.1 shows, the NMR spectra of tyrosine and phenylalanine are considerably simplified by exchange of all but the C(2)H and C(6)H hydrogens. The spec-

trum of tryptophan can be made equally simple by fully deuteriating the amino acid (for example by acid catalysis [8,9]) and back exchanging the C(2)^{2}H for hydrogen. Similar spectral simplification should result in the spectra of peptides and proteins biosynthesized from the amino acids.

The experiments with *N*-acetyl-tyrosine amide have demonstrated that the amino and carboxylate groups are not necessary for the exchange process to occur. In addition, the mildness of the exchange conditions allows the possibility that intact peptides and proteins might be selectively substituted with ^{2}H or ^{3}H directly. This will have considerable potential both for NMR and for other studies.

Acknowledgements

The skilled technical assistance of J. T. LaFontaine and N. K. Sueoka in carrying out catalyst preparations and exchange reactions is gratefully acknowledged. We wish to thank Professor D. B. Melville for the suggestion and helpful advice in using deuterized Raney nickel to catalyze ^{2}H exchange. This work was supported in part by Grant AM21739-01 from the US Public Health Service. The NMR experiments were performed at the NMR Facility for Biomolecular Research, Francis Bitter National Magnet Laboratory, MIT. The NMR facility is supported by grant no. RR00995 from the Division of Research Resources of the National Institutes of Health and by the National Science Foundation under contract no. C-670.

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