Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXXVI, No. 2

APPLICATION OF SOLID STATE CATALYTIC HYDROGEN ISOTOPE EXCHANGE TO THE TRITIUM LABELING OF LYSOZYME

Anton V. Filikov[1,2,3,*], John R. Jones[1], Nikolai F. Myasoedov[2] and E. Sally Ward[3]

[1] *Chemistry Department, University of Surrey, Guildford GU2 5XH, Surrey, UK*
[2] *Institute of Molecular Genetics, Russian Academy of Sciences, Kurchatov Sq., Moscow 123182, Russia*
[3] *Cancer Immunobiology Center and Department of Microbiology, University of Texas, Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-8576*

Solid state catalytic hydrogen isotope exchange has been employed to label hen egg lysozyme with tritium. Optimization of reaction conditions so that amino acids and peptide bonds remained intact led to a tritiated product with 97% of the original enzymatic activity and 94% radiochemical purity. The specific activity when using a T_2:H_2 mixture of 1:1000, was 16 mCi·mmol^{-1}. It is suggested that the currently adopted approach may have wide applications for other proteins able to tolerate lyophilization conditions without loss of activity.

Key words: isotope exchange, proteins, solid state hydrogenation. tritium, deuterium

INTRODUCTION

The most widely used methods [1] for incorporating tritium into organic compounds are (1) catalytic hydrogenation and dehalogenation using T_2 gas, (2) catalytic hydrogen isotope exchange with T_2 gas and (3) hydrogen isotope exchange using tritiated water. The latter is the most versatile of the three methods, as acids, bases, metals and enzymes can all be employed as catalysts and the number of compounds labeled by this method is very large [2]. The need to synthesize a precursor makes the use of hydrogenation and dehalogenation reactions less attractive and also expensive for fairly complex compounds such as short peptides. The corresponding precursors for long peptides and proteins cannot be produced, i.e. the only choice is exchange methods. As far as solutions are concerned such methods can only provide rather low levels of radioactivity, usually of the order of mCi·mmol^{-1} for peptides, and are not useful for proteins since the latter tend to lose their biological activity after the reaction. An alternative is Solid State Catalytic Hydrogen Isotope Exchange (SSCHIE) which is a variant of Solid State Catalytic Hydrogenation (SSCH).

Although SSCH reactions [3] were first observed in 1964, the subsequent decade witnessed relatively few additional examples, all of them dealing with inorganic compounds. Only in 1976 was its first application to organic compounds reported [4], and only within the last few

---

* to whom correspondence should be addressed (at University of Texas)

CCC 0362–4803/95/020179–07

Received 2 September, 1994
Revised 5 September, 1994

years has the work become familiar to a wider audience [5-11, 15]. Its main thrust has been towards the tritiation of selected groups of organic compounds. Complementary studies on SSCHIE [5] and Solid State Catalytic Dehalogenation (SSCD) have also been reported [4].

Solid state catalytic hydrogenation is a reaction carried out in a vessel containing a hydrogenation catalyst, the initial compound (as a powder) and gaseous hydrogen. SSCH is based on hydrogen spillover - the penetration of activated hydrogen from the metal catalyst into the organic compound phase. If diffusion of activated hydrogen species into the compound phase is a rate limiting step, a gradient of spillover-hydrogen is formed and the kinetics of the reaction demonstrates unusual features for the reaction systems with parallel reactions of isotope exchange and hydrogenation [9, 10] and for isotope exchange reactions resulting in formation of two or more isotopomers [11]. Since the SSCH reaction proceeds in a crystalline environment, many hydrogenation reactions are prohibited or partially suppressed due to steric reasons: some reactions fail to proceed because newly-formed chemical bonds cannot "fit" into the rigid crystal structure, others because the crystal structure does not allow hydrogen spillover particles to approach the necessary group. The crystalline environment acts to preserve the structure of the molecule, and it can prevent the hydration of multiple bonds, hydrogenolysis reactions, etc. Because SSCH and SSCHIE reactions are carried out in the absence of a solvent higher temperatures can be employed and for many compounds very high levels of radioactivity can be introduced into the products.

One would expect that the crystalline environment affects solid-state catalytic hydrogen isotope exchange to a lesser degree, because these reactions do not modify the structure of the molecule. It has been demonstrated for thymine [6] that a decrease in the temperature lowers the rates of solid-state hydrogenolysis and double-bond hydration reactions more rapidly than for isotope exchange, i.e. all side-reactions are virtually eliminated at lower temperatures.

In theory the application of SSCHIE provides an opportunity of introducing a tritium label into proteins with minimal by-product formation. The purpose of the current work is therefore to develop an experimental procedure for the SSCHIE tritiation of proteins in such a way that the product requires no further purification after the reaction has taken place. The development of the method for proteins should be preceded by the search for the reaction conditions ensuring the highest possible specific radioactivity at still acceptable by-product level formation for the amino acids, which contribute mostly to by-product formation. Furthermore, reaction conditions should be chosen to ensure stability of the peptide bond. This method could be useful for the tritiation of proteins and peptides for use in NMR studies. In particular, tritiated peptides could be used in isotope-edited NMR [12] to analyze peptide-ligand interactions.

EXPERIMENTAL

Materials. Hen egg white lysozyme (M.W. ca. 14300) was obtained from Sigma and the amino acids and peptide used from Aldrich. Platinum black was prepared according to [13], and 5% $Pd/CaCO_3$, 5% Pd/C, 5% $Pd/BaSO_4$, 5% Pd/Al_2O_3 were from commercial sources (Aldrich, BDH, and Engelhard).

NMR spectroscopy. The ^1H and ^2H nmr spectra of the amino acids were recorded on a Bruker AC 300 MHz spectrometer with D_2O or H_2O as solvent. A ROESY spectrum [14] of Gly-Gly-Gly was obtained in 10% D_2O with 3 mg of the peptide on Varian VXR500. It was recorded as 100 t_1 increments (64 transients/increment) with 1024 complex points in t_2. Quadrature detection was achieved using hypercomplex data acquisition. The time-domain data were multiplied by a Lorenzian-to-Gaussian apodization function (FELIX gb=0.25; lb=-8) and a 50^o shifted sine-bell function was applied in t_2. Alpha proton resonances were assigned relying on their ROESY cross peaks with amide proton resonances.

Procedures for SSCHIE reaction. In previous studies using thymine [15] four methods have been used for reaction mixture preparation and the best of these was employed in the current investigation. The compound (1-10 mg) was first dissolved in water at a level of 20 $mg \cdot ml^{-1}$ and the solution then lyophilized. The resulting powder was then mechanically mixed with a

determined amount of catalyst. Several milligrams of the mixture were then placed in the reaction vessel (10 cm^3) and attached to the gas line before evacuating and passing D_2 gas or T_2:H_2 mixture (1:1000) up to a pressure of ca. 300 torr. At this stage the reaction vessel was thermostated for a pre-determined time. On completion and cooling 0.5 ml of water was added to the reaction vessel and the resulting suspension centrifuged. The clear solution was pipetted off and rotary evaporated to remove labile deuterium or tritium. The procedure for removing labile tritium from lysozyme is discussed below.

Enzymatic activity assay. The enzymatic activity assay for lysozyme [16] was performed with 0.2 mg/ml lyophilized *Micrococcus luteus* (Sigma) dissolved in 40 mM sodium phosphate buffer (pH 6.2). A test solution (20-50 ul) was added to the substrate solution to make a final volume of 2 ml. The decrease in absorbance (at 520 nm) was measured at 25°C. Lysozyme disrupts the polysaccharide components of bacterial cell walls which results in the decrease in their absorbance.

Electrophoretic analysis. The purity of the lysozyme after the reaction was determined by continuous non-denaturing 15% polyacrylamide gel electrophoresis [17] in 40 mM sodium phosphate buffer, pH 6.2. Non-denaturing electrophoresis was used instead of the more common sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) since the mobility in non-denaturing electrophoresis depends not only on molecular weight as in SDS-PAGE case, but also on the net charge of the native molecule and on its size and shape, i.e. various modifications of the protein molecule could be detected with this method in contrast to SDS-PAGE, which could only detect a change in molecular weight. The presence of by-products in the preparation of tritiated lysozyme was assessed both visually and radiometrically. For visual assessment the gel was stained with an aqueous solution containing 0.1% Coomassie blue R-250, 50% methanol and 7.5% acetic acid and destained with 5% methanol and 7.5% acetic acid in water. In order to determine the distribution of radioactivity in the lane, the gel was cut into small pieces and placed into standard vials with dioxane scintillator overnight, and the radioactivity determined by scintillation counting.

RESULTS AND DISCUSSION

Selection of the most effective catalyst. In order to choose the catalyst ensuring the maximum level of isotope exchange the activity of five commercial and one prepared catalysts were compared for the reaction of deuterium exchange with glycine at 165°C. As can be seen from Table 1 the most active catalyst was 5% $Pd/CaCO_3$. We assume here that relative reaction rates for isotope exchange and by-product formation do not depend on catalyst activity. According to the current notion concerning SSCH the catalyst serves as a source of active hydrogen species, which migrate into the organic compound phase, and all reactions proceed not on the catalyst surface but within the bulk of the compound, i.e. relative rates of reactions must be determined

TABLE 1. The comparison of performance of several catalysts in SSCHIE reaction of deuteration of glycine at 165°C

catalyst	glycine/catalyst ratio, w/w	incorporation of deuterium atom/molecule
Pt black	1:10	<0.1
Pt black synthesized according to [13]	1:10	0.7
5% $Pd/CaCO_3$	1:50	0.8
5% Pd/C	1:50	<0.1
5% $Pd/BaSO_4$	1:50	<0.1
5% Pd/Al_2O_3	1:50	<0.1

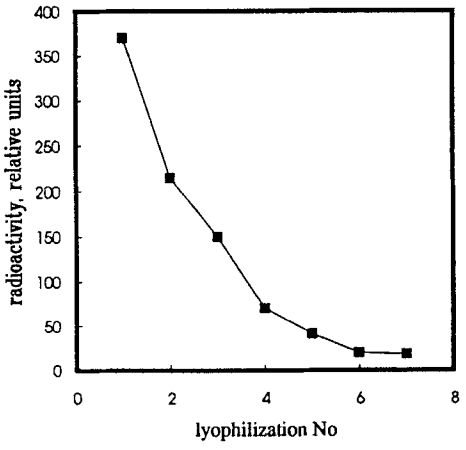

Fig. 1. Radioactivity of lysozyme solution after tritiation by SSCHIE and repeated lyophilization.

primarily by the nature of the compound. Nevertheless, the nature of the catalyst could influence the reaction indirectly through possible mobility of catalyst particles or change of physical structure of the reaction mixture during the course of the reaction, etc. Here we neglect all these possible effects and do not carry out screening of catalysts for each compound studied and subsequently use 5% $Pd/CaCO_3$ in all tritiations.

The development of a method for removing labile radioactivity from the protein after SSCHIE reaction. Usually in order to remove the labile radioactivity (tritium in positions other than CH) from a compound a lyophilization procedure is employed. In the case of lysozyme, it proved that only after several lyophilizations the radioactivity level for the protein attained a constant value (fig. 1). This behavior is probably due to slow exchange of the labile hydrogens which are hidden inside the protein globule and only accessible for solvent molecules when the globule changes conformation.

An alternative approach proved to be much more effective. Passage of the preparation of lysozyme through a very short (ca. 1 cm high) column of Sephadex G-25 (a media for gel filtration) followed by lyophilization ensured complete removal of labile radioactivity. Further repetition of lyophilizations resulted in no reduction of the sample radioactivity. The effective exchange of labile tritium with water during the movement of the protein solution through the column occurs for reasons that are not clear and require further investigation.

Selection of reaction conditions ensuring amino acids and peptide bonds remain intact. Proteins can be labeled to a specific radioactivity of 10-20 mCi/mmol when using T_2:H_2 gas mixture (1:1000). This means that if the label is uniformly distributed among all protein molecules, about 30% to 60% of molecules are labeled. The molecule of lysozyme contains hundreds of protons. There exists the possibility that the reaction proceeds only in a very thin reaction layer adjacent to the catalyst surface. In this case only a small proportion (can be several per cent) of molecules are labeled. All tests of enzymatic activity can only ensure accuracy worse than 3%, i.e. in this case there are no means to test enzymatic activity of the labeled molecules themselves. Because of this, the optimization of reaction conditions for labeling enzymes should be preceded by optimization for constituent amino acids and optimization for the peptide bond. The following amino acids were chosen: glycine, L-lysine, DL-phenylalanine, DL-histidine, L-tryptophan, L-methionine, which cover most of labile functional groups in amino acids. Stability of the peptide bond was studied using a Gly-Gly-Gly peptide. The reactions were carried out with deuterium. Incorporation of deuterium was monitored by ^2H NMR and chemical yield and purity by proton

NMR. The experimental results are shown in Table 2. The assignment of the resonance for the peptide was done on the basis of amide proton to alpha proton cross peaks in a ROESY spectrum.

On the basis of these results there can be derived "safe" temperatures for amino acids in SSCHIE reaction, i.e. maximum temperatures ensuring ca. 98% chemical purity after reaction (Table 3). It can be seen from the table that a temperature of 92°C (exponential interpolation of data for methionine) ensures stability of all tested amino acids. Since the amino acids studied appear to cover all labile functional groups in native amino acids, it can be estimated that 92°C is an optimal temperature for peptides and small proteins.

TABLE 2. Analytical data on the compounds deuterated using SSCHIE reaction

Compound	Reaction conditions, temp., time	Ratio of chemical purity after reaction to that before, %	Chemical yield, %	Deuterium incorporation, atom/molecule	Position of the label	Abundance at this position, %
Glycine	85°C, 30 min	99%	96%	<0.01		
	165°C, 30 min	99%	70%	0.8	α	40%
L-Lysine	110°C, 32 min	100%	98%	<0.01		
	131°C, 30 min	100%	96%	0.02	α	2.1%
DL-Phenyl-alanine	20°C, 1010min	99%	56%	<0.01		
	80°C, 35 min	99%	60%	0.2	α	20%
	95°C, 15 min	99%	66%	0.08	α	8%
	140°C, 34 min	94%	52%			
	158°C, 37 min	<10%	<5%			
DL-Histidine	86°C, 31 min	98%	68%	0.17	2	17%
	106°C, 44 min	98%	48%	0.4	2	40%
L-Tryptophan	112°C, 32 min	99%	97%	0.19	α	9%
L-Methionine	85°C, 30 min	99%	86%	<0.01		
	117°C, 30 min	81%	73%	0.16	α	16%
Gly-Gly-Gly	110°C, 44 min	100%	27%	<0.01		
	150°C, 46 min	100%	17%	0.08	gly-1	4%
				0.12	gly-2	6%
				0.08	gly-3	4%

TABLE 3. Maximum temperatures ensuring ca. 98% chemical purity after SSCHIE reaction.

Compound	Maximum temperature (°C)
Glycine	>165
Lysine	>131
Phenylalanine	95
Histidine	106
Tryptophan	>112
Methionine	92
Gly-Gly-Gly	>150

Choosing optimal reaction conditions for labeling lysozyme. 5 mg of lysozyme were dissolved in 0.5 ml of water, lyophilized and mechanically mixed with 250 mg of 5% $Pd/CaCO_3$. This reaction mixture was used for all experiments with lysozyme. After the reaction with T_2:H_2 (1:1000) gas, 0.5 ml of water was added to the reaction mixture. The suspension was centrifuged, the supernatant was passed through 1 cm height column of Sephadex G-25 and lyophilized. The lyophilized lysozyme was dissolved in 40 mM sodium phosphate buffer, pH 6.2 and analyzed for enzymatic activity and for purity by electrophoresis. Visually there were no traces of impurities for all samples on the electrophoretic gel. The distribution of radioactivity along each lane was quantitated and the radiochemical purity was calculated as a ratio of the radioactivity of the spot of lysozyme to the total radioactivity of the lane. The experimental data are shown on Table 4.

It can be seen that a temperature of 80°C and reaction time of 30 min result in only a 3% decrease in enzymatic activity as well as a reasonable radiochemical purity and specific radioactivity. The enzymatic activity assay cannot detect lowering of enzymatic activity of labeled molecules themselves in the case of a very thin reaction layer, but since the reaction temperature of 80°C is considerably lower than the "safe" temperature for amino acids and peptide bonds (92°C) we can reliably assume that labeled molecules are enzymatically active.

Although we employed the current approach only to labeling of lysozyme, it should be possible to use it for other peptides and proteins which tolerate lyophilization without loss of activity. Lysozyme is known to be a very stable protein and the method may therefore not be suitable for proteins that are substantially less stable. In order to assess the lower limit of the probability of obtaining a damaged protein molecule after reaction one should add the probabilities of damage for the amino acid residues that are present in the protein, as well as the probabilities of damaging peptide bonds and disulphide bridges. Because of this, for bigger proteins lower temperatures will have to be used, which will result in a lower level of radioactivity per amino acid residue being incorporated. Another obstacle which can be expected for larger proteins is the lowering of chemical yield. For some reason a proportion of the compound becomes insoluble after the SSCH reaction due to adherence to the catalyst. For bigger proteins this effect may become significant due to aggregation of partly denatured molecules.

TABLE 4. Experimental data for tritium labeling of lysozyme using a T_2:H_2 gas mixture (1:1000)

Reaction conditions, temp./time, °C/min	Chemical yield %	Specific radio-activity, mCi/mmol	Enzymatic activity, %	Radiochemical purity, %
20 30	93	1.4	95	95
80 30	90	16	97	94
100 30	85	71	58	90
115 30	81	117	26	81
162 30	36	162	18	74

ACKNOWLEDGMENTS

The authors wish to thank NATO (A.V.F., N.F.M.) and NIH (A.V.F., E.S.W.) for financial support. Thanks are due to Dr. M.D. Frank-Kameneckaia (Institute of Molecular Genetics, Russian Academy of Sciences) for assistance with electrophoretic analyses.

REFERENCES

1. Evans E.A. Tritium and Its Compounds, Butterworths, London, 1974.
2. Jones J.R., in: Isotopes: Essential Chemistry and Applications (ed. J.A. Elvidge & J.R. Jones), The Chemical Society, London, Chapter 10, Hydrogen Isotope Exchange Reactions.
3. Khoobiar S.J., J. Phys. Chem., 68, 411 (1964).
4. Myasoedov N.F., Marchenkov N.S., Mikhailov K.S., in "Organicheskie Soedineniia Mechenye Radioactivnymi Isotopami, Proc. Intern. Symp., Marianske Lazne, May 1976", p. 275, CZKAE, Praha, 1977.
5. Filikov, A.V., Myasoedov, N.F. J. Radioanal. Nucl. Chem. Lett. 85: 373 (1984).
6. Zolotarev Yu. A., Kozic V.S., Zaitsev D.A., Dorochova E.M. and Myasoedov N.F. J. Label. Comp. & Radiopharmaceuticals, 29: 507 (1991).
7. Zolotarev Yu. A., Kozic V.S., Dorochova E.M. and Myasoedov N.F. J. Label. Comp. & Radiopharmaceuticals, 29: 997 (1991).
8. Zolotarev Yu. A., Kozic V.S., Dorochova E.M., Zaitsev D.A. and Myasoiedov N.F. Synthesis and Applications of Isotopically Labelled Compounds 1991, Proceedings of the Fourth International Symposium, Toronto, Canada, 3-7 September 1991", Elsevier, Amsterdam, p. 687, 1992.
9. Filikov A.V., Myasoedov N.F. J. Phys. Chem., 93, 2502-2506 (1989).
10. Filikov A.V., Myasoedov N.F. J. Catal, 130, 569-576 (1991).
11. Filikov A.V., Myasoedov N.F. J. Phys. Chem., 98, 3924-3926 (1994).
12. Griffey R.H., Redfield A.G. Quart. Rev. Biophys., 19, 51-82 (1987).
13. Willstatter R., Waldschmidt-Leitz E., Ber., 54, 113 (1921).
14. Neuhaus D., Williamson M. The Nuclear Overhauser Effect in Structural and Conformational Analysis. VCH Publishers, Inc. 1989, pp. 313-327.
15. Filikov A.V., Myasoedov N.F. in:"Synthesis and Applications of Isotopically Labelled Compounds, Proceedings of the Third International Symposium, Innsbruck, Austria. 17-21 July 1988", pp. 549-552, Elsevier, Amsterdam, 1989.
16. Parry R.M., Chandon R.C., Shahani K.M. Proc. Soc. Exp. Biol. 119, 384-386 (1965).
17. Protein Structure, a Practical Approach (ed. by T.E. Creighton) IRL Press, Oxford, pp. 225-230 (1990).