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Muon Spectroscopy Applied to Biological Systems: A Study of Thiyl Radicals, RS·

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Thiyl radicals (RS·) are formed when positive muons (μ^+) are implanted into solutions of thiocarbonyl compounds (C = S) in ethanol, tetrahydrofuran or formamide solvents. A solvent dependence is found, which reflects changing electronic interactions and overall conformations, suggesting that the properties of RS· radicals may be dependent on the polarity (philicity) of the environment in which they are formed.

Keywords: Muon, muonium, thiyl radicals, antioxidant, environment, electronic structure, radio-tracer

INTRODUCTION

Thiyl Radicals are widely implicated in biological systems,[1,2] for example as H-atom donors which repair free radical damage on bio-molecules, and in the nitrosylation of protein thiols; it has also been suggested that they are involved in the thiol-induced enhancement of oxidative modification of low-density lipoprotein, although the mechanistic details remain speculative. It has been proposed that thiyl radicals might be detoxified in cells in the presence of glutathione (GSH) and superoxide dismutase. The postulated mechanism involves the initial oxidation of GSH to GS· by superoxide or other radicals, and the resulting glutathionyl radicals react with GS^- to form the glutathione radical anion ($GSSG^{-}\cdot$). $GSSG^{-}\cdot$ then regenerates superoxide by the reduction of dioxygen, and so initiates a chain reaction. Superoxide dismutase ultimately dismutates superoxide to H_2O_2 and O_2, thereby acting as a "radical sink".[1] Results from recent work suggest that thiyl radicals are detoxified by thiol-specific enzyme in bacterial systems, but it is not clear whether such an enzyme exists in mammalian cells.[1]

As in any mechanistic evaluation of a transient intermediate, it is highly desirable to obtain some details of electronic structure and geometry: while thiyl radicals may be detected indirectly in ESR/spin-trapping ex-

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periments,[3] they have never been studied directly by ESR in solution, although they may be trapped and thereby measured in solid matrices.[4]

Consideration of the electronic structure of an RS· radical which, formally, is orbitally degenerate has led to the conclusion that they should be undetectable in solution by ESR in consequence of their highly anisotropic g-tensor[4] resulting in extremely broad lines; moreover, the high reactivity of RS· radicals towards their precursors will favour the detection of product radicals such as $RS\text{-}SR_2$·[5] or RSS·.[6]

Given these difficulties, which we prefer to avoid, we now introduce an altogether different approach for the study of RS· radicals which uses positive muons as a radioactive probe of magnetic interactions in radicals.

BACKGROUND TO THE TECHNIQUE

It is the purpose of this paper to communicate our preliminary results, and thereby indicate the potential of muon spin rotation (TF-μSR) spectroscopy[7,8] for studying free radicals related to those extant in biological systems, and since this approach will be novel to the great majority of biochemists, the following overview of the method itself should prove helpful.

Muons, fundamental sub-atomic particles, are a component of cosmic radiation but are formed for research purposes by bombarding a target of suitable material (carbon or beryllium) with medium energy protons. Among the products of the ensuing nuclear reactions that occur are pions (binding components of nuclei): these decay on a nanosecond timescale to muons (*via* $\pi^+ \text{—>} \mu^+ + \nu_\mu$) which may be implanted into matter, for the study of the magnetic properties of solid state, liquid phase and gas phase processes. Depending on the charge of the pion, either positive or negative muons are formed in their decay: negative muons find application, *inter alia*, in the promotion of nuclear fusion, while their positive counterparts apply particularly to Chemistry, Biology, Materials and Catalysis research.[9]

While muons are leptons, to use the classification of Nuclear Physicists, and belong formally to the "electron" category, as chemical species positive muons behave as protons, forming a bound state with a negative electron which is normally dubbed "muonium" (μ^+e^-): muonium is equivalent to a normal protium atom (p^+e^-) and indeed shows the chemical properties of a *light* hydrogen atom, undergoing both H-atom abstraction and addition reactions. It serves well, therefore, as a radioactive probe of H-atom chemistry[10] and of processes involving free radicals since the latter are readily formed by addition of muonium to unsaturated organic substrates, e.g.

$$H_2C=CH_2 + Mu\cdot \rightarrow Mu-CH_2CH_2\cdot,$$
$$R_2C=S + Mu\cdot \rightarrow R_2C(Mu)-S\cdot.$$

Thus labelled, these radicals are characterised by a single pair of lines in the TF-μSR spectrum, on the application of a high transverse magnetic field, which represent the –1/2, +1/2 m_s spin combination with the muon (m_μ) states: these occur at the precession frequencies from muons which experience the sum of the applied and (–1/2, + 1/2 m_s) hyperfine magnetic fields. The muon-electron hyperfine coupling constant is obtained from the difference between the high (ν_2) and low (ν_1) frequencies for each radical: $A_\mu = \nu_2 - \nu_1$; as the coupling increases, for a given magnetic field, the frequency ν_2 increases, while concomitantly that ν_1 first decreases, reaches zero and then increases due to a sign change in the transition: in the latter limit the coupling is obtained from the sum of the frequencies: $A_\mu = \nu_2 - (-\nu_1)$. An analysis of the data may be made corresponding to each frequency as described below.*

EXPERIMENTAL

The μSR experiments were carried out using the μE4 beamline at the Paul Scherrer Institute, Villigen, Switzerland. Solutions (20 wt%) were prepared each of thioacetamide, thiobenzamide, N,N-dimethylthioformamide, ethylene trithiocarbonate and 3-ethyl-2-thioxo-4-oxazolidinone in ethanol, tetrahydrofuran and formamide (all reagents were purchased from Aldrich and were used without further purification) and were deoxygenated at 10^{-4} mmHg, before being sealed into 35mm o.d. thin-walled pyrex ampoules. In each experiment, the sample was maintained in an applied magnetic field of 0.2T, while being irradiated with positive muons with a momentum of 85MeV/c; data of 20–40 million decay events were accumulated and processed as described in the footnote, yielding the muon-electron coupling constants.

Semi-empirical M.O. calculations were carried out on the John Moores University Vax Cluster, using the PM3 Hamiltonian,[11] as available in the MOPAC6 package.[12] The geometry was optimised using either a UHF or RHF Hamiltonian. In the single point calculations, with the RHF approximation, a configuration interaction was allowed over an active space consisting of the two highest doubly occupied and the singly occupied together with the lowest three unoccupied levels.

RESULTS AND DISCUSSION

In this preliminary study, we have investigated the temperature dependences of the muon coupling in thiyl radicals $R_2C(Mu)$-S·, formed by the addition of muonium to the following thiocarbonyl compounds (C = S): thioacetamide, thiobenzamide, N,N-dimethylthioformamide, ethylene trithiocarbonate and 3-ethyl-2-thioxo-4-oxazolidinone, as a function of solvent (tetrahydrofuran, ethanol, formamide). Our results are summarised under the following headings.

Temperature Dependences

In all cases, other than the radical formed from thiobenzamide, the muon coupling was found to increase with decreasing temperature. This confirms our original conclusion[13] that the equilibrium conformation of C(Mu)-S· radicals is that in which the C-Mu bond eclipses the density axis of the nominally sulphur centred $3p_z$ orbital. While this is also true for the adduct of ethylene trithiocarbonate, the coupling is in all solvents lower than that of all thiyl radicals other than that from thiobenzamide (*vide infra*), and is due to the increased weighting of conformations in which vacant orbitals on the sulphur atoms interact with the unpaired electron orbital, thus providing stabilisation.

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* Muons decay to positrons ($\mu^+ \rightarrow e^+ + \nu_e + \bar{\nu}_\mu$) on a microsecond timescale, which are detected using scintillation counters, and counted using fast electronics; the decay events are accumulated in 4 data histograms. Generally, the histogram is of the form

$$N(t) = N_0\left\{B + \exp(-t/t_\mu)[1 + F(t)]\right\},$$

where N_o is a normalisation factor, roughly equal to the number of counts in the first channel (t = O), B is the background fraction (usually <1%), t_μ is the muon lifetime and F(t) reflects the time dependence of the muon spin polarisation. For experiments in which a number of frequencies are obtained (as in the present case), F(t) is the sum of contributions of the form

$$F_j(t) = A_j \exp(-\lambda j t)\cos(\omega j t + \phi j),$$

corresponding to a muon precession at a specific frequency νj, for which Aj is the asymmetry (amplitude), λj the relaxation rate, and provides a measure of chemical reactions or physical relaxation processes, and ϕj is the initial phase. The data are analysed by fitting directly the latter expression to the experimental data in Fourier space, which yields the parameters $Aj, \lambda j, \omega j, \phi j$ for each frequency.

Effect of Solvent

This may be illustrated with reference to the following table of muon-electron hyperfine couplings/MHz obtained at 300K.

Sample	THF	Ethanol	Formamide
Thioacetamide	487	464	435
Thiobenzamide	201	197	181
N,N-dimenthylthioformamide	501	500	482
Ethylene Trithiocarbonate	345	355	346
3-Ethyl-2-thioxo-4-oxazolidinone	419	420	409

It is clear that, in all cases, the coupling is reduced in formamide solution: this, we propose, is caused by specific solvation of the thiyl Sz atom, in the manner RSz—C = O, which will reduce the energy of the 3pz level relative to the C-Mu s and s*, levels, which are responsible for hyperconjugative spin-transfer to the muon. The *difference* between ethanol and THF is generally small, other than for thioacetamide which may relate to solvation of the NH2 group by H-bonding in ethanol solution, and a change in conformational "control" of the radical.

The value is very similar in *all* media for the radical derived from ethylene trithiocarbonate, and the effect of formamide may be offset by additional solvation of the *other* sulphur atoms, thus reducing their C-S σ* orbital energies concomitantly with that of the C-S. $3p_z$ level, leading to a reduced net effect.

In the biological context, these results demonstrate a solvent dependence, which reflects changing electronic interactions and overall conformations, suggesting that the properties of RS· radicals may depend on the polarity (philicity) of the environment in which they are formed, i.e. be determined by their location in either hydrophobic or hydrophilic regions.

The Thiobenzamide Adduct: $PhC(Mu)(NH_2)S\cdot$

We feel that this radical deserves the honour of a separate heading, since its behaviour is quite unlike that of the rest. Clearly, its coupling is reduced to *ca* 40–50% of the other typical values; moreover, in all three solvents the temperature dependence is *negligible*, implying either that the rotation about the C-S· bond is free or that the conformation is largely *fixed*, by a substantial torsional barrier. Even were the highest value measured—that for DMTF in THF (501 MHZ)—to represent the fully eclipsed conformation (i.e. the maximum coupling), free rotation would give *half* this, on the normal $\cos^2\theta$ basis. The appreciable increase in the coupling on cooling, however, tells that the eclipsed limit has not been reached and so the half-value of *ca* 250MHz is certainly an underestimate of that for *free* rotation. Since the measured coupling is far lower even than this low estimate, we reject this possibility and propose that the conformation is essentially fixed, and lies in a potential significantly below that of other torsional states.

In order to understand this effect, we have carried out *semi-empirical* M.O. calculations on the radicals $PhC(Mu)(NH_2)S\cdot$ and $MeC(Mu)(NH_2)S\cdot$. The results so far, which we are currently trying to refine, predict that the coupling in the former radical should be reduced to about 50% of that in the latter, and that its equilibrium geometry eclipses the phenyl group with the sulphur SOMO, in the manner of an incipient bridging structure. Since this geometry decreases the hyperconjugative overlap between the SOMO and the C-Mu bond, at least qualitatively, the fall in the muon coupling is explained.

Substituent Effects

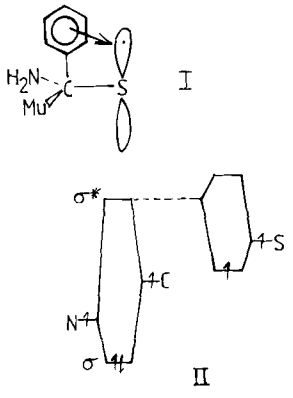
While the influence of phenyl and sulphur substituents, in the adducts of thiobenzamide and ethylene thiocarbonate, may be rationalised respectively in terms of an incipient phenyl bridging structure (I) and interactions with σ* C-S orbitals, substituent effects in the remaining radicals are more subtle. We feel that the salient common structural feature of the ad-

ducts from N,N,-dimethylthioformamide, thioacetamide and 3-ethyl-2-thioxo-4-oxazolidinone is the presence of a β-nitrogen atom, the relative electron releasing power of which decreases along this series, belonging to, respectively, Me_2N, H_2N and N-C=O groups.

Considering the competing interaction between the S $3p_z$ SOMO and both the C-Mu and C-N bond σ* orbitals (II), the energy of the N-C σ* will be raised in the stronger C-N σ-bond as the nitrogen atom becomes more electron releasing: therefore, the S-C-N σ* interaction will be weaker, and that with the C-Mu bond will dominate further the conformation of the radical and hence raise the coupling. Qualitatively, this would account for the fall in the muon coupling along the series: Me_2N, H_2N, N-C=O.

FUTURE AIMS

Having arrived at some conclusions regarding environmental and substituent effects in thiyl radicals, we now wish to probe some aspects of reactivity. The importance of the reactivity of thiyl radicals concerns their role in biological systems,[1,2] in which they are known to undergo a variety of reactions including electron transfer, hydrogen abstraction and addition reactions with biological substrates and xenobiotics. We emphasise that it is not possible to use ESR spectroscopy to detect thiyl radicals in fluid solution because of extreme relaxation effects which broaden the spectral lines beyond detection; thus the technique is intrinsically inappropriate for the study of the kinetics of reactions involving them. We plan to investigate the kinetics of thiyl radical reactions with substrates implicated in biological systems; for instance cholesterol, trilinolein and trilinolenin, in connection with the currently vexed question of the role of thiyl radicals in lipid peroxidation,[1] for which kinetic data, as obtained in a very direct way using μSR,[7,8] would be highly valuable.



STRUCTURE I

Acknowledgements

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