Synthesis of two series of cysteine-specific spin labels containing a ^{15}N substituted nitroxide

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SUMMARY

Synthesis of two series of spin labels with a cysteine-reactive group, either maleimide or methanethiosulfonate, and a ^{15}N substituted nitroxide separated at variable distance is reported. In the synthesis, the cysteine-reactive groups were first incorporated into the target molecule and the ^{15}N substituted nitroxide was introduced in the last step via a mild reaction without destroying the cysteine-reactive groups. The synthetic routes described here are applicable to the synthesis of spin labels with either ^{15}N substituted or "normal" ^{14}N nitroxide. Spin labeling of cysteine residues in rhodopsin by the newly synthesized spin labels was also demonstrated.

Key Words: Spin label, EPR, Maleimide, methanethiosulfonate, nitroxide, rhodopsin.

INTRODUCTION

Spin labeling has found important applications in biological studies.[1-3] Recent studies have shown that structural and kinetic information of proteins can be obtained by spin labeling technique.[4,5] Normally, stable nitroxides with the free radical on the bond

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CCC 0362–4803/95/121173–09

Received 23 May 1995
Revised 19 July 1995

between the common ^{14}N isotope of nitrogen and oxygen atoms are used for spin labeling studies. This "normal" nitroxide gives a three-line EPR spectrum due to the interaction between the nuclear spin of the ^{14}N and the free radical. To study proteins with spin labeling technique using this nitroxide as a free radical, concentration greater than ten micro molar of the spin labeled protein is required to obtain an EPR spectrum with reasonable signal-to-noise ratio. Such a high protein concentration requirement is one of the limiting factors in designing spin labeling experiments. One way of increasing the signal-to-noise ratio in spin labeling experiment is to modify the nitroxide.

It has been shown that the substitution of ^{14}N by ^{15}N and proton by deuterium on the nitroxide ring greatly increased the signal-to-noise ratio as compared with the "normal" nitroxide under the same conditions in spin labeling studies.[6] In addition, ^{15}N substituted nitroxide gives a two-line spectrum due to the interaction of the ^{15}N nuclear spin with the free radical. The new two-line spectrum occurs at different positions from their ^{14}N counterpart. Thus, in addition to the increased sensitivity, the ^{15}N substituted nitroxide also offers a tool for new experimental design. For example, the electron-electron double resonance experiment is greatly simplified when a pair of the "normal" ^{14}N and the ^{15}N substituted nitroxide are used. Though the synthesis of spin labeled lipids with the ^{15}N substituted nitroxide have been intensively studied,[7-9] the synthesis of protein spin labeling reagent with a ^{15}N substituted nitroxide has been less investigated. As a consequence, the application of spin labeling protein with the ^{15}N substituted nitroxide is hampered by the lack of the commercial availability and the lack of simple routes to the synthesis of these important reagents.

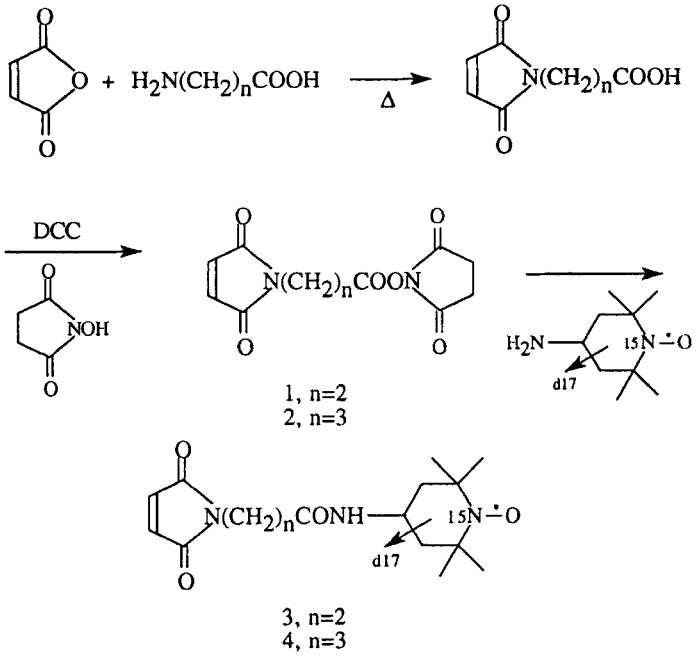
Here I report the synthesis of two series of ^{15}N substituted nitroxides with two kinds of cysteine-reactive groups, methanethiosulfonate and maleimide, respectively. These two series of spin labeling reagents enable us to obtain a spin labeled protein with the nitroxide at variable distance from its attaching site on the protein. The variable distance of the nitroxide from protein gives us a control of the mobility of the nitroxide and thus the EPR line shape.

RESULTS AND DISCUSSION

The sulfhydryl group of cysteine residues on a protein is mostly targeted for protein modifications because of its nucleophilicity. Spin labels with maleimide or methanethiosulfonate reactive group has been widely used to modify cysteine residues in a protein.[10] Spin labels with methanethiosulfonate reactive group are more selective than the maleimide reactive group in reacting with sulfhydryl group. The spin labeling by methanethiosulfonate is reversible upon addition of reducing reagents such as dithiothreitol, whereas the spin labeling by maleimide group is non-reversible. Each class of the above reagents has its own advantage and applications. Thus, the synthesis of both classes of spin labels with ^{15}N substituted nitroxide is described here.

The synthesis of spin labels with maleimide reactive group and ^{15}N substituted and perdeuterated nitroxide is shown in Scheme 1. The ω-amino substituted fatty acids with different carbon chain lengths are commercially available. The ω-amino group was modified with maleic anhydride to generate a reactive maleimide group. The carboxyl

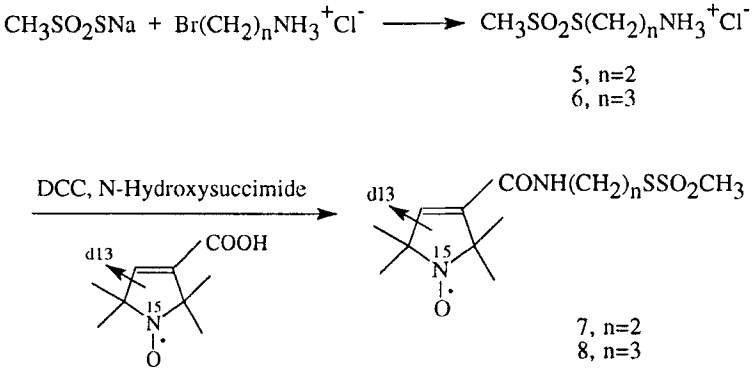
Scheme 1



group at the opposite end was then activated by DCC in the presence of N-hydroxysuccinimide (1,2)[11,12]. The activated carboxyl group coupled with 4-Amino-1-^{15}N-1-oxy-2,2,6,6-tetramethyl-piperidine-d_{17} to yield the target molecules (3,4). This series of spin labels has varying carbon chain length between the ^{15}N substituted nitroxide and the reactive maleimide group depending on the carbon chain length of the starting ω-amino substituted fatty acid.

The synthetic procedure here is different from the synthetic procedure of the similar compound with a "normal" ^{14}N nitroxide in the sequence of introducing the nitroxide group and the maleimide group. Normally, a nitroxide is attached to the carboxyl end of a ω-amino substituted fatty acid first and the maleimide group is attached to the amino end at the last step.[10] This procedure avoided exposing it to nucleophilic attack by other reagents by assembling the labile cysteine-reactive group in the last step. However, the total yield is lower comparing with the present procedure with regard to the nitroxide since it must go through all the subsequent reactions. Since the ^{15}N spin labels are extremely valuable, the present procedure increased at least twice of the yield than the normal procedure with regard to the starting ^{15}N substituted nitroxide by assembling it in the last synthetic step. The key to the strategy of current procedure is to use a mild reaction condition in assembling the nitroxide to the target molecules to avoid destroying the labile cysteine-reactive groups. The only structural difference between these new spin labels with the ^{15}N isotope and the similar spin labels with the ^{14}N isotope is that the amide bond is facing at opposite direction in these molecules.[10]

Scheme 2



Scheme 2 shows the synthesis of spin labels with ^{15}N substituted and perdeuterated nitroxides and methanethiosulfonate reactive group. The starting compound, ω-bromo substituted amines with varying carbon chain length are commercially available. Addition of sodium thiosulfonate to the ω-bromo substituted amines yields amines with methanethiosulfonate group at the ω-position (5,6). The peptide bond coupling between these methanethiosulfonate substituted amines with 1-^{15}N-1-oxy-2,2,5,5-tetramethyl-3-pyrrolin-d_{13}-3-carboxylic acid gives rise to the final compounds (7,8). These new kinds of spin labels have methanethiosulfonate reactive group and the ^{15}N substituted nitroxide separated at varying distance. The distance between the cysteine-reactive group and the nitroxide can be varied by using different starting ω-bromo substituted amines. Again, this procedure gives a high yield of product with regard to the starting ^{15}N substituted nitroxide by assembling the nitroxide group at the last step of synthesis.

In the synthesis, the structures of all the intermediate compounds were confirmed by NMR and high resolution mass spectrum. The final spin labeled products contain free radicals and hence their NMR spectrum cannot be obtained. High resolution mass spectrometer was used to confirm the atomic composition of these compounds. Furthermore, these spin labels have been shown to react specifically with cysteines on rhodopsin and cysteines on mutated cholicine and sharp two-line EPR spectra were obtained in both cases.[5,13,14]

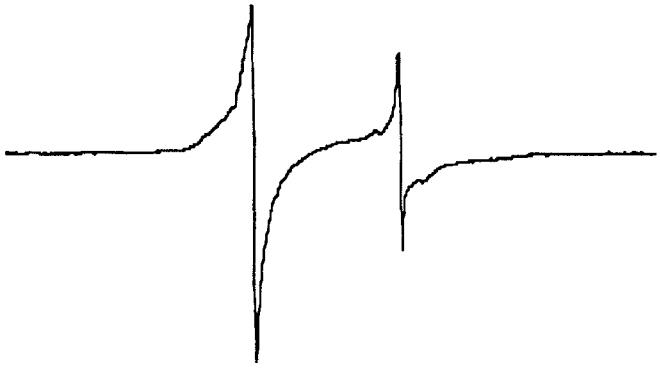


Figure 1. EPR spectrum of Compound 8 after it was attached to residues Cys316 and Cys140 of rhodopsin. The sample was equilibrated with nitrogen and the spectrum was taken at room temperature. Scan Width = 100 G; Power = 1 mW; Modulation Amplitude = 0.5 G; Receiver Gain = 1000.

An example of the use of these spin labels in labeling rhodopsin is presented here. It has been shown that the two reactive cysteine residues Cys140 and Cys316 on rhodopsin can be spin labeled and the line shape of the resulting EPR spectra depend on the distance of the nitroxide from the attaching site[15]. Similar results were also observed with the spin labels reported here. When compound 4 was used to label rhodopsin, one population of sharp EPR spectrum with nitroxide in isotropic motion was observed, which is ideal for electron-electron double resonance experiment[13]. However, at least two populations of nitroxide with anisotropical motions were observed when compound 8 was used to label rhodopsin (Figure 1). When spin labels shorter than compound 8 was used, more complicated spectrum was observed[14].

In conclusion, methods were developed to synthesize cysteine-specific spin labels with ^{15}N substituted and perdeuterated nitroxide. By assembling the cysteine reactive group to the target molecules at the first step and coupling the ^{15}N substituted and perdeuterated nitroxide at the last step, two classes of cysteine-reactive spin labels were obtained with high yield. This was accomplished by choosing mild reaction conditions in the last step to couple the nitroxide and the intermediate compounds that contain labile cysteine-reactive groups without destroying them. By using these series of spin labels, the distance between the protein attaching site and the ^{15}N substituted and perdeuterated nitroxide can be varied. Such series of cysteine-specific spin labels with ^{15}N substituted and perdeuterated nitroxide are extremely valuable for biological studies. The procedure described here is also suitable for synthesizing the corresponding common non-isotope reagent by using the corresponding nonisotope nitroxide starting material.

EXPERIMENTAL

4-Amino-1-^{15}N-1-oxy-2,2,6,6-tetramethylpiperidine-d_{17} and 1-^{15}N-1-oxy-2,2,5,5-tetramethyl-3-pyrrolin-d_{13}-3-carboxylic acid were obtained from MSD ISOTOPES. 2-Bromoethylamine hydrobromide, 3-bromopropylamine hydrobromide, β-alanine, 4-aminobutyric acid were obtained from Aldrich (Milwaukee, WI). N-(3-maleimidopropanyl-oxy) succinimide and N-(4-Maleimidobutyryloxy) succinimide were synthesized according to the method of Rich et al.[11] and Tanimory et al.[12]. Sodium

methanethiosulfonate was synthesized according to the method of Kenyon and Bruce[16]. 3-(N-Morpholino)propane sulfonic acid (MOPS) was obtained from Sigma (St. Louis, MO). Rod outer segment membranes were prepared as described[13,17]. NMR was done on a Bruker AM360 spectrometer. Mass spectra was done on a VG ZAB-SE spectrometer. EPR was done on a Varian E-109 EPR spectrometer operating at X-band and fitted with a two-loop-one-gap resonator.

Synthesis of spin label 4-(3'-Maleimido-propamido)-1-^{15}N-1-oxy-2,2,6,6-tetramethylpiperidine-d_{17} (3) and 4-(4'-Maleimido-butyramido)-1-^{15}N-1-oxy-2,2,6,6-tetramethylpiperidine-d_{17} (4)

The synthesis of compound 3 and 4 were analogous and only the synthesis of compound 4 is described here. Compound 2 (34 mg, 0.18 mmol) and 4-Amino-1-^{15}N-1-oxy-2,2,6,6-tetramethylpiperidine-d_{17} (50.4 mg, 0.18 mmol) were mixed in 1 ml of THF and stirred for 12 hours at room temperature. The solvent was then removed by rotary evaporation and 3 ml of methylene chloride was added to dissolve the residue. The organic solution was washed twice with 3 ml of water. The organic layer was dried with anhydrous magnesium sulfate and the product was purified by flash liquid chromatography on 30-60 mesh silica. The column was presaturated with ether and eluted with chloroform/methanol/water at 65:25:4. The yellow nitroxide band was collected, giving 52 mg (yield 82%) of yellow semi-solid. The compound was pure as judged by TLC on silica gel eluting with chloroform/methanol/water at 65:25:4. The compound composition was confirmed by high resolution mass spectra with a molecular ion M/e=354.2935, a value with a -2.5% deviation from that expected for compound 4 ($C_{17}H_9D_{17}N_2{}^{15}NO_4$).

Synthesis of 2-amino-ethylmethane-thiosulfonate (5) and 3-amino-propylmethane-thiosulfonate (6)

Syntheses of compound 5 and 6 were analogous and only the synthesis of compound 6 is described here. 3-bromo-propylamine hydrobromide (4.38 g, 0.02 mmol) and sodium thiosulfonate (2.68g, 0.02 mmol) in 40 ml of absolute ethanol were refluxed for 6 hours. The reaction mixture was cooled to 0^{o}C and the sodium chloride was filtered out as a white precipitate. The yellowish filtrate was concentrated to 20 ml and left to stand at

4°C overnight. The white precipitate was collected by filtration and washed with ethanol. The resulting white solid was further purified by recrystallization with ethanol. Light beige colored fine crystal (2.63g, 52% yield) were obtained with m.p.125-126°C.
NMR: The ^{1}H NMR spectrum (D_2O as solvent) showed four peaks at δ2.05(2H,m), δ3.02(2H,t), δ3.20(2H,t), δ3.40(3H,s). The ^{13}C NMR spectrum (D_2O as solvent) showed four peaks at δ19, δ25, δ31, and δ42.

Synthesis of S-(1-^{15}N-1-oxy-2,2,5,5-tetramethylpyroline-d_{13}-3-carboxamido-ethyl) methanethiosulfonate (7) and S-(1-^{15}N-1-oxy-2,2,5,5-tetramethyl-pyroline-d_{13}-3-carboxamidopropyl)methanethiosulfonate (8)

The syntheses of compound 7 and 8 were analogous and only the synthesis of compound 8 is described here. The above synthesized 3-amino-propylmethane-thiosulfonate (6) (0.22 mmol), N-hydroxysuccinimide (25.3 mg, 0.22 mmol), N,N-dimethylaminopyridine (27 mg, 0.22mmol), and 1-^{15}N-1-oxy-2,2,5,5-tetramethyl-3-pyrrolin-d_{13}-3-carboxylic acid (39 mg, 0.22mmol) were dissolved in 1 ml DMF and cooled to 0°C. DCC (46 mg, 0.22mmol) in 0.5 ml DMF was then added to this solution with stirring. The solution was warmed to room temperature and continually stirred overnight. The solution was then removed by rotary evaporation. Chloroform (2 ml) was added to the residue and the resulting solution was washed with saturated sodium bicarbonate solution followed saturated sodium chloride solution. The organic layer was dried by anhydrous sodium sulfate. The organic solvent was removed and the residue is purified with flash liquid chromatography on a 30-60 mesh silica gel eluting with 15:1 chloroform/methanol. The yellow nitroxide band was collected giving 46 mg compound 12 (yield 66%). The product was pure as judged by TLC. The product was confirmed by high resolution mass spectra with a molecular ion M/e 349.1853, a value with a deviation of -3.2% from the theoretical value for compound 9 ($C_{13}H_{10}NO_4D_{13}{}^{15}NS_2$).

Spin labeling of rhodopsin and EPR measurement.

Rod outer segment membranes (containing about 2 mg/ml rhodopsin) in 20 mM MOPS buffer, pH = 7.0 and 100 mM stock solution of compound 4 or compound 8 in methanol were mixed and the resulting mixture was incubated with gentle stirring in dark at 30°C for 2 hours. The ratio of the spin label to rhodopsin was approximately 20 to 1 and the

amount of methanol never exceeded 1% v/v. The excess of compound 4 or compound 8 was then removed by washing three times with 50 ml of 20 mM MOPS at pH = 7. The resulting membrane pellets with spin labeled rhodopsin was then transferred to a TPX capillary and place in a loop-gap EPR resonator and equilibrated with nitrogen. The EPR spectra were taken at room temperature.

ACKNOWLEDGMENT

This research was supported by NIH Grant EY05216, a grant from Research to Prevent Blindness, and the Jules Stein Professor endowment to Prof. W. Hubbell. The author would like to thank Prof. Hubbell for his financial support and research guidance.

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