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One- and two-dimensional proton NMR studies of cys-102 *S*-methylated yeast isozyme-1 ferricytochrome *c*

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ABSTRACT The effect of *S*-methylating cysteine-102 (cys-102) (SH → $SSCH_3$) of yeast isozyme-1 (iso-1) ferricytochrome *c* has been studied using proton NMR spectroscopy. COSY, NOESY, and one-dimensional nuclear Overhauser effect (NOE) difference spectroscopies have all been used. The NMR spectrum of this derivative is very similar to that of native yeast iso-1 ferricytochrome *c*. The advantage of using the cys-102 *S*-methylated derivative is that it is unable to spontaneously dimerize in solution, like native iso-1 monomer does. This makes the derivative a simple, ideal protein for long NMR experiments. This work yields many proton resonance assignments for *S*-methylated yeast iso-1 monomer and confirms all of the assignments for iso-1 monomer that were previously made using only the one-dimensional NOE method.

INTRODUCTION

Saccharomyces cerevisiae (Baker's yeast) contains two major cytochromes *c*, termed isozyme-1 and isozyme-2 (1, 2). Isozyme-1 (iso-1) is prevalent in yeast mitochondria and, of particular interest to us, is the fact that it is the natural redox partner for yeast cytochrome *c* peroxidase (3). Iso-1 differs from iso-2 in that the primary sequence of iso-1 contains an unusally reactive cysteine at position 102 (4, 5). This cysteine allows the monomer (native) iso-1 to undergo spontaneous in vitro dimerization to form the disulfide linked iso-1 dimer (3–5). Evidence has been presented that such dimerization, as well as chemically modifying cysteine-102 (cys-102) with certain reagents (3–5) results in significant protein conformational changes. Furthermore, the rate of dimerization of monomer iso-1 ferricytochrome *c* under solution conditions optimized for proton NMR spectroscopy is fast enough to complicate interpretation of NMR experiments that require long time periods for data acquisition.

One solution to this problem is to create proteins with a point mutation replacing cys-102 (6, 7). A chemically simpler approach is chemical modification of cys-102, but as described above, many such chemical modifications result in significant changes both in spectra and folding properties and, by inference, in structure (3–5, 8).

In this work we present proton NMR results for yeast iso-1 ferricytochrome *c* that has been modified by *S*-methylation at cys-102. A combination of proton COSY, NOESY, and one-dimensional nuclear Overhauser effect (NOE) difference spectroscopy leads to an increased number of proton resonance assignments and to a direct comparison with published NMR spectra of native yeast iso-1 (3). This comparison reveals that native iso-1 and *S*-methylated iso-1 display very similar spectra, which indicates that large conformational changes are not apparent for this derivative, as they are for other derivatives (3). Further, the COSY experiments confirm many of the assignments previously made for yeast iso-1, using solely NOEs.

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MATERIALS AND METHODS

Iso-1 cytochrome *c* from *S. cerevisiae* (Type VIIIA) was purchased from Sigma Chemical Co. (St. Louis, MO). This yeast iso-I cytochrome *c* was modified at cys-102 with methyl methanethiolsulfonate (MMTS, Aldrich Chemical Co., Milwaukee, WI) by a slight modification of the procedure of Ramdas et al. (9). 100 mg of iso-1 were dissolved in 3 ml of 0.01 M potassium phosphate, pH 7.6, 0.2 M β-mercaptoethanol (Sigma Chemical Co.) and allowed to stand at room temperature for 15 min. The solution was then passed through a column (2.5 × 40 cm) containing sephadex G-25-30 resin (Sigma Chemical Co.) equilibrated with 0.01 M glycine that was adjusted to pH 7.8 with NaOH. To the effluent (~100 μM cytochrome *c*) was added a volume of aqueous 0.1 M MMTS such that the molar ratio of MMTS to protein was 4.0 (10). The reaction was carried out at 4°C with stirring for 30 min. The reaction mixture was loaded onto a column (1 × 10 cm) of (BioRex-70; Bio-Rad Laboratories, Richmond, CA) resin that was equilibrated with 0.05 M potassium phosphate, pH 7.2, and washed with several column volumes of the same buffer containing a stoichiometric amount of potassium ferricyanide (i.e., 1:1 molar ratio of ferricyanide ion to protein). After washing the column with several volumes of 0.05 M potassium phosphate, pH 7.2 (minus potassium ferricyanide), the protein was eluted with 0.25 M potassium phosphate, pH 7.2. The resulting protein did not react with 5,5'-dithio-2-nitrobenzoate (DTNB) in an assay for free sulfhydryls (11, 12) and was monomeric on sodium dodecyl sulfate gels run in the absence of sulfhydryl reagents.

Proton NMR spectra were obtained on either a 361-MHz spectrome-

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ter (General Electric Co., Fremont, CA) or an AC 250-MHz spectrometer (Bruker Instruments, Inc., Billerica, MA). The COSY and NOESY pulse sequences used were the standard experiments in the instruments' program library. Typical two-dimensional parameters on the Bruker Instruments, Inc. spectrometer were as follows: 90° pulse = 9 μs, carrier located on water, t_1 = 256 increments and t_2 = 512 points. The t_1 dimension was zero filled to give a 512 × 512 final size. The window function was a sine bell in both dimensions. Data were symmetrized to eliminate ridges in the diamagnetic envelope and a 20-kHz spectral width was used. In NOESY experiments mixing times of 30 and 50 ms were used, whereas total recycle times of 0.5–1.5 s were used. One-dimensional NOE difference spectroscopy was carried out as previously described (3) using 30- and 50-ms irradiation times and total recycle times of 0.5–1.0 s. Observed shifts are reported relative to the residual internal HDO resonance that was assigned a shift of 4.63 ppm. Spectra were taken at either 21, 24, or 25 ± 1°C, in D_2O solutions consisting of 10 mM KNO_3, adjusted to pD 6.5 with 1 M DCl in D_2O. The protein was not lyophilized, but exchanged into D_2O using an Amicon pressure ultrafiltration cell. No correction for isotope effect was made and the meter reading of the D_2O solutions is reported as pD, with adjustments carried out using either DCL or NaOD (both MSD isotopes).

RESULTS AND DISCUSSION

Fig. 1 shows the 361-MHz proton NMR spectrum of cys-102 *S*-methylated yeast iso-1 ferricytochrome *c*. Some of the assignments made in this work are shown, although more complete assignments are given in Table 1. These assignments for methyl modified iso-1 were made using a combination of phase sensitive NOESY, phase sensitive COSY, one-dimensional NOE difference spectroscopy, comparison to previously published native yeast iso-1 ferricytochrome *c* (3, 13), and comparison to horse ferricytochrome *c* (14). Representative spectra are presented and discussed below.

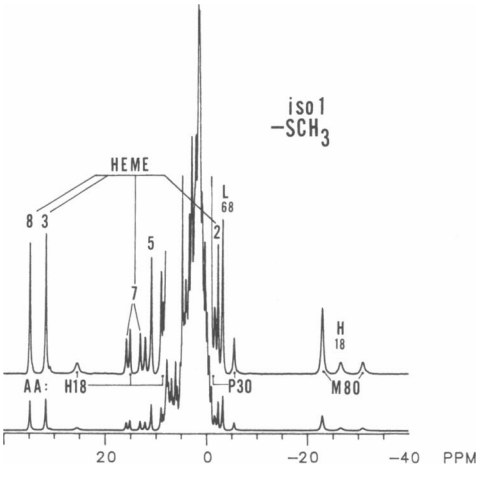

FIGURE 1 361 MHz proton NMR spectrum of cys-102 *S*-methylated yeast iso-1 ferricytochrome *c* with some assignments for amino acids and heme indicated. Conditions: 10 mM KNO_3, pD 6.5, 2 mM [cyt], 25°C.

TABLE 1 Proton resonance assignments for S-CH_3 modified yeast iso-1 ferricytochrome *c* and comparison with native monomer iso-1

Assignment‡	Shift	
	Native	Cys-102 modified
Heme:		
8-CH_3	34.7	34.7
3-CH_3	31.4	31.5
5-CH_3	10.8	10.8
7α-CH_2	15.8	15.7
	12.8	12.9
7β-CH_2	1.17	1.13
	−0.40	−0.46
4β-CH_3	2.68	2.67
2β-CH_3	−2.47	−2.42
2α-CH		−0.96
Amino acid:		
phe-10	8.51	8.47
His-18, β-CH_2	14.9	14.9
	8.82	8.82
His-18, α-CH	8.82	8.82
His-18, δ-CH	24.9	25.3
His-18, ε-CH	−26.5	−26.5
Gly-29, α-CH_2*§	−3.4	−3.22
		−0.60
Pro-30, δ-CH_2	−5.75	−5.60
	−1.71	−1.70
Pro-30, γ-CH		−0.50
Trp-59	7.36	7.40
Leu-68, δ-CH_3	−3.41	−3.35
Leu-68, γ-CH		0.54
Met-80, ε-CH_3	−23.4	−23.1
Met-80, γ-CH_2	−30.7	−30.9
	2.50	2.41
phe-82, Φ-ring	5.97	5.96
phe-82, β-CH*	3.71	3.70

Shifts reported in parts per million relative to internal HDO (=4.63 ppm), in 10 mM KNO_3, 25 ± 1°C, pD = 6.5.
‡New assignments for native iso-1 indicated by asterisk, previous assignments from references 3 and 12. All assignments for *S*-methylated iso-1 are new and the result of this work.
§Approximate shift in native iso-1 for Gly-29 resonance overlapping Leu-68 δ-CH_3 resonance.

The first major point that we wish to make is the remarkable similarity in assigned resonance shifts of native iso-1 (3, 13), with the cys-102 *S*-methylated iso-1, as reflected in Table 1. The close similarity in the spectra of these two proteins is emphasized by the fact that, where they occur, observed shift differences are, with few exceptions, only ~0.1 ppm.

In contrast, the iso-1 dimer and iso-1 monomer modified at cys-102 with 5,5′-dithiobis(2-nitrobenzoate) (iso-1-TNB; 3) exhibit large spectral changes compared with native iso-1 monomer (3). Individual shift differences of up to 1.6 ppm are induced by dimerization, or TNB modification.

These results indicate that *S*-methylated iso-1 mono-

mer is a close conformational mimic of the unmodified monomer protein. Although the chemical shift differences between native iso-1 and cys-102 *S*-methylated iso-1 are small, they are readily reproducible, thereby indicating that the two derivatives in the oxidized form have very similar, but not strictly identical structures. Recent protein folding studies on both native iso-1 monomer and *S*-methylated iso-1 monomer have found essentially no differences in the unfolding behavior of the two (9, 15). This result is generally consistent with our conclusion and also suggests that NMR is more sensitive to slight structural differences between the two protein forms.

There are two other advantages of using the cys-102 *S*-methylated iso-1 monomer in spectral studies. (*a*) Modification of cys-102 by the procedure of Smith et al. (10) requires rather simple chemistry, which is reversible in the presence of sulfhydryl reagents. (*b*) Disulfide dimerization is eliminated and there is very little tendency of the modified protein to autoreduce over time.

The second major point of this work is to compare the one-dimensional NMR assignments of yeast iso-1 (3) with the combination of one-dimensional and two-dimensional methods employed here. In this case, this is a comparison of simple two-dimensional and one-dimensional methods specifically applied to low spin, paramagnetic ferriheme proteins which is important because one-dimensional NOE methods have so far been widely applied to elucidating dipolar connectivities in a variety of ferriheme proteins (3, 16–19).

For assignments of magnetically inequivalent geminal and vicinal partner protons, a straightforward method for confirming assignments made solely by NOE experiments is through the use of COSY. COSY experiments elucidate J-coupling connectivities even for the paramagnetically broadened resonances (Figs. 2 and 3), where J-couplings are not resolvable in the one-dimensional spectrum (Fig. 1). However, for paramagnetic proteins the short T_2s that cause broad resonances also result in distortion and reduction of intensity in phase sensitive COSY cross-peaks, apparently due to cancellation of overlapping antiphase parts of the cross-peaks. One consequence of this effect is difficulty in extracting accurate coupling constants from COSY data taken on paramagnetic proteins. A more general difficulty is simply detecting COSY cross-peaks because the net effect is reduction in cross-peak intensity. This result becomes more severe

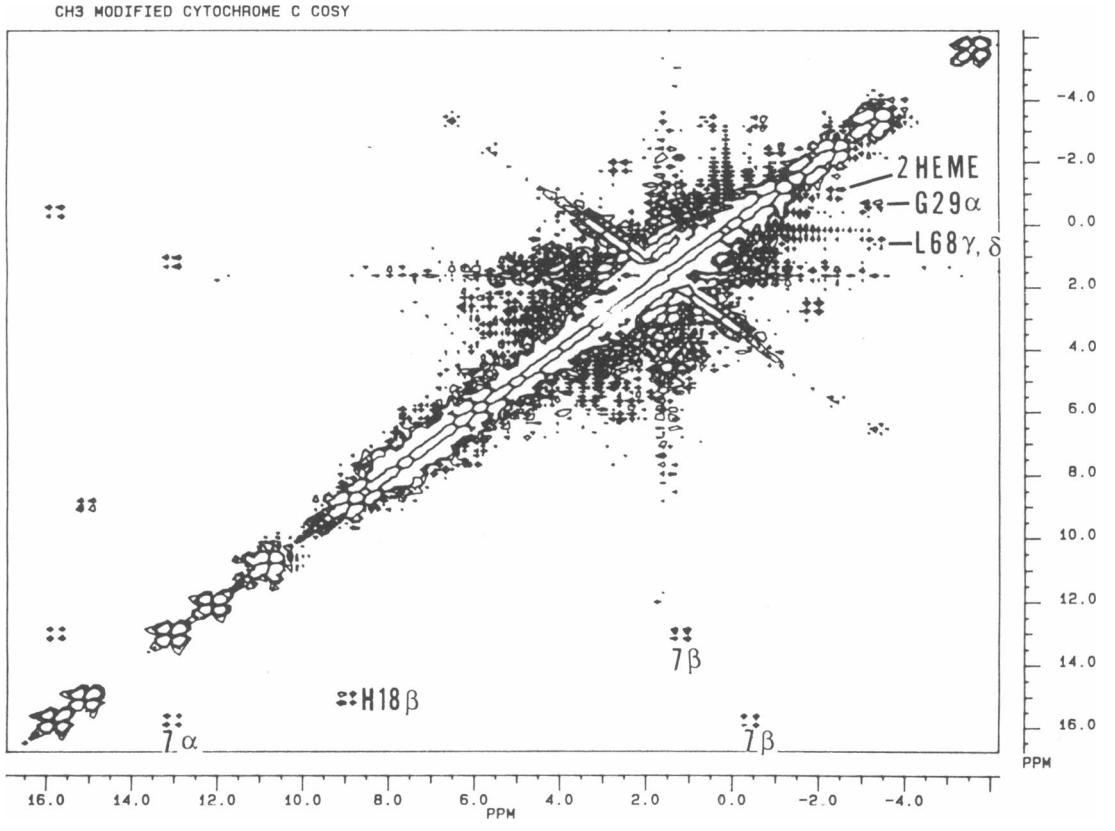

FIGURE 2 Selected region of the 250 MHz proton COSY spectrum of cys-102 *S*-methylated yeast iso-1 ferricytochrome *c*. Conditions identical to Fig. 4.

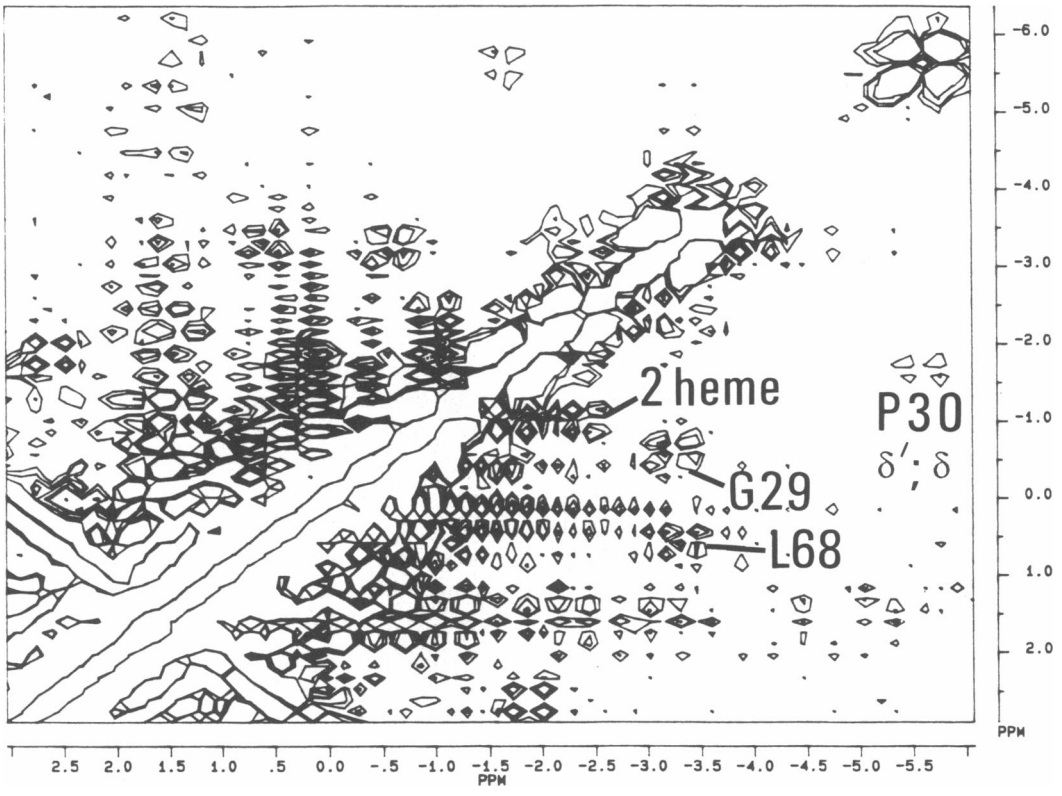

FIGURE 3 Expanded region of the 250 MHz proton COSY spectrum of cys-102 *S*-methylated yeast iso-1 ferricytochrome *c* plotted at a lower contour level than for Fig. 2 to show the pro-30 cross-peak. Conditions identical to Fig. 4.

at higher fields and for larger proteins (unpublished data) due to anomalous line broadening as a result of the so-called "Curie spin relaxation" mechanism (20). For this reason, in part, our COSY spectra (Figs. 2 and 3) were run at 250 MHz.

The NOESY spectrum presented in Fig. 4 and the one-dimensional NOE difference spectra presented in Fig. 5 (additional data not shown) contain connectivity patterns identical to those observed in the one-dimensional NOE experiments previously published for native yeast iso-1 and iso-2 monomers (3, 16). In the contour plot shown in Fig. 4 NOESY cross-peaks between hyperfine resonances are identified and labeled. These lead to essentially identical assignments for the hyperfine-shifted resonances of native iso-1 and *S*-methylated iso-1, as noted on the spectra (Figs. 4 and 5) and in Table 1. However, unlike the one-dimensional NOE difference spectra that produced connectivities from the broad, far upfield resonances assigned to the met-80 ϵCH_3 and γCH_2 protons into the diamagnetic region (0–10 ppm), no NOESY cross-peaks among these resonances have been detected in our two-dimensional spectra. We infer from the limited number of assignments for met-80 so far produced for horse ferricytochrome *c* (14), that this is a general problem for the broad resonances of fast relaxing nuclei.

The NOE connectivity patterns of ferric iso-1 (3), ferric iso-2 (16), and *S*-methylated ferric iso-1 (this work) are essentially identical. Due to this, more extensive assignments of any one of these three proteins should be directly applicable to the other two. Furthermore, it was of general interest, as well as being instructive, to compare the results from one-dimensional NOE difference spectra and appropriate slices of the full two-dimensional NOESY spectrum. Such a comparison for the heme 3-CH_3 (slice in Fig. 5 *C*, or irradiation in Fig. 5 *B*) is shown in Fig. 5. In this case care was taken to employ NOESY mixing times identical to irradiation times used for the one-dimensional NOE spectra. Even doing this it is our conclusion from several such comparisons that some connectivities are more readily observed using the one-dimensional NOE experiment rather than NOESY. In addition to the met-80 case noted in the previous paragraph, a further example is the one-dimensional selective detection of connectivity between heme methyl-3 (31.5 ppm; Fig. 5) and phe-82 (5.96 ppm; Fig. 5). Detecting this NOE and using it to monitor phe-82 dynamics has been fundamental to our work with cy-

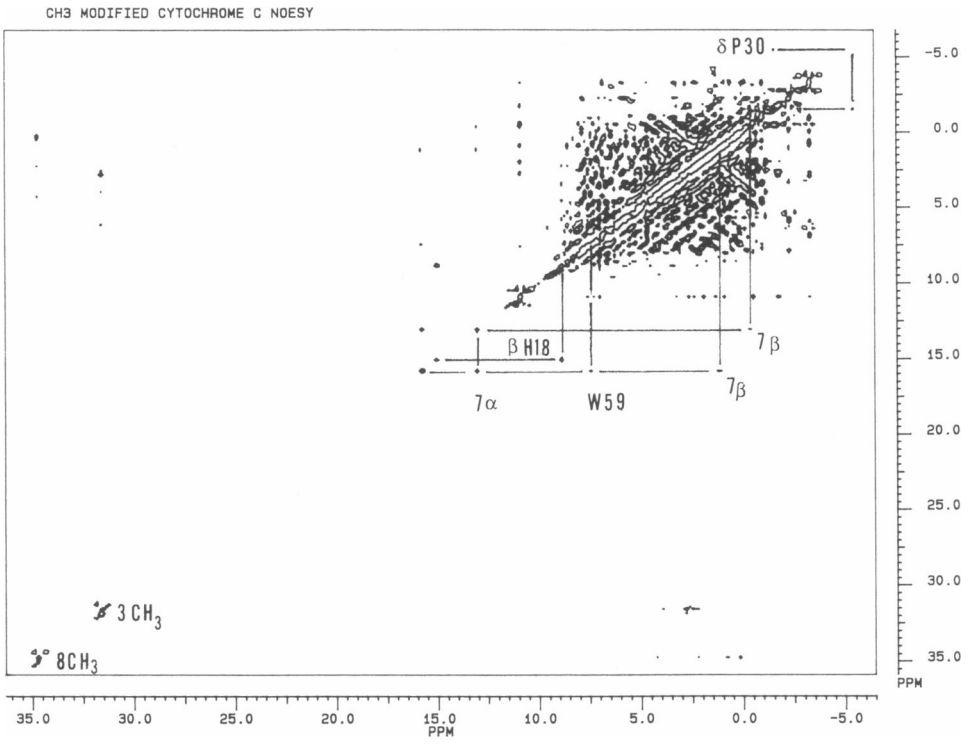

FIGURE 4 Full 250 MHz proton NOESY contour map for cys-102 *S*-methylated yeast iso-1 ferricytochrome *c*. Conditions: 10 mM KNO_3, pD 6.5, 2 mM [cyt], 24°C.

tochrome *c* peroxidase/ferricytochrome *c* redox complexes (21, 22). Examination of the NOESY contour plot in Fig. 4 shows no cross-peak characteristic of this connectivity, whereas the connectivity is clearly shown in the one-dimensional experiment in Fig. 5 *B*. Further, a comparison of the NOESY slice through the heme 3-CH_3 and the one-dimensional NOE difference spectrum corresponding to irradiation of the heme 3-CH_3 shown in Fig. 5 *C* (mixing time identical to irradiation time for Fig. 5 *B*) indicates the greater sensitivity, selectivity, and resolution of the one-dimensional experiment, which, incidentally, took only ~15% of the time required for the complete two-dimensional experiment. Even in the NOESY slice shown in Fig. 5, a 5.96-ppm peak is not obvious. Factors that contribute to this state of affairs in specific cases such as this include the better signal-to-noise ratio achievable in reasonable time for the selective one-dimensional experiment and, perhaps, the fact that different coherence pathways operate in the two types of experiments.

It is clear however, that a combination of one-dimensional (where applicable) and two-dimensional experiments is the most efficient procedure for obtaining assignments in paramagnetic proteins compared with the one-dimensional NOE method alone, especially at the low fields to which we are currently limited. In this respect, COSY spectra provide crucial confirmation of NOE based assignments of geminal and vicinal partners. An example of this is the diastereotopic pro-30 δ,δ' (CH_2) resonances that were identified by one-dimensional NOEs for native iso-1 monomer (3). The NOESY spectrum in Fig. 4 shows a contour cross-peak from one pro-30 proton at −5.60 ppm to its geminal partner at −1.70 ppm. Note that the pro-30 δ-CH peak that should be on the diagonal at −5.60 ppm is not observed at this mixing time (30 ms), or for others that we have tried. Although a COSY cross-peak between this pair is not observed at the contour level of Fig. 2, it is seen in Fig. 3, at a lower contour level, and confirms the assignment of these resonances to a pair of magnetically inequivalent geminal partners.

Assignments made specifically by two-dimensional methods for the *S*-methylated yeast ferric iso-1 that were ambiguous, or not detected, in our previous work on native ferric iso-1 (3) and ferric iso-2 (16) are shown in Table 1. These include the heme 2α, pro-30 γ, and leu-68 γ protons. In addition we have tentatively assigned the two α protons of gly-29 to single proton resonances at −3.22 ppm (overlapping leu-68 δ-CH_3) and at −0.6 ppm. These gly-29 assignments are based on the fact that gly-29 single protons are detected in this region in horse ferricytochrome *c* (−4.49 and −0.98 ppm; 13) and the strong COSY cross-peak between the −3.22 and −0.6 ppm resonances, appropriate for geminal partners. The

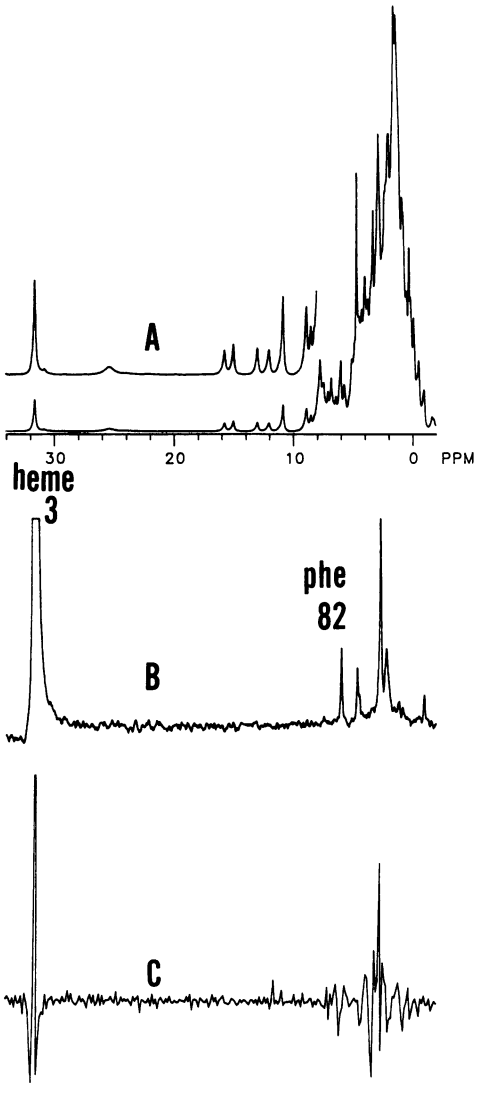

FIGURE 5 (*A*) Selected region of the 361 MHz proton absorption NMR spectrum of cys-102 *S*-methylated yeast iso-1 ferricytochrome *c*. (*B*) One-dimensional proton NOE difference spectrum with on-resonance irradiation at the heme 3-CH_3 and an irradiation time of 30 ms. (*C*) Row slice of the two-dimensional NOESY spectrum taken with a 30-ms mixing time. Displayed spectral regions are identical in Figs. *A–C*. Conditions identical to Fig. 1.

nearly identical proton spectra of native and methyl modified yeast iso-1 (as well as yeast iso-2) implies that these assignments are identical in each, even though we have not specifically indicated them for the native protein in Table 1.

SUMMARY

In this work we have shown the following. (*a*) *S*-methylation of cys-102 in native yeast iso-1 ferricytochrome *c* produces a protein which has a proton NMR spectrum very similar to native monomer iso-1. This result indicates that the conformations of the two proteins are very similar, which along with inhibition of dimerization and autoreduction, makes the *S*-methylated derivative highly suitable for NMR exeriments carried out under a broad range of conditions. (*b*) COSY, NOESY, and one-dimensional NOE experiments on *S*-methylated ferric iso-1 have provided assignments that are essentially identical to those previously reported for native iso-1. The COSY spectra presented here confirm the previous assignments made solely by one-dimensional NOE difference spectroscopy for native iso-1, and provide several additional assignments. (*c*) For paramagnetic heme proteins, and in particular for high molecular weight protein complexes with short T_2s, where detecting behavior of only a limited number of key amino acids is important, one-dimensional methods, where applicable (i.e., for hyperfine shifted resonances), can provide superior sensitivity and greater efficiency than full two-dimensional experiments.

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