Inhibition by Cyclopropylamine of the Quinoprotein Methylamine Dehydrogenase Is Mechanism-Based and Causes Covalent Cross-Linking of α and β Subunits†

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ABSTRACT: Cyclopropylamine acted as a mechanism-based inhibitor of the quinoprotein methylamine dehydrogenase from *Paracoccus denitrificans*. The protein-bound quinone cofactor of this enzyme was rapidly reduced by addition of a stoichiometric amount of cyclopropylamine, but this compound did not serve as a substrate for the enzyme in the steady-state kinetic assay. Time-dependent inactivation of the enzyme by cyclopropylamine was observed only in the presence of a reoxidant. Saturation behavior was observed, and values of K_I of 3.9 μM and K_{inact} of 1.7 min^{-1} were determined. Enzyme inactivation was irreversible, as no restoration of activity was evident after gel filtration of methylamine dehydrogenase which had been incubated with cyclopropylamine in the presence of a reoxidant. The inactivated enzyme exhibited an altered absorption spectrum. Electrophoretic analysis of inactivated methylamine dehydrogenase indicated that covalent cross-linking of the α and β subunits of this $\alpha_2\beta_2$ oligomeric enzyme had occurred and that the quinone cofactor had been modified. A mechanism for this inhibition is proposed which is based upon the data presented and is consistent with the available structural information on methylamine dehydrogenase.

Methylamine dehydrogenase from *Paracoccus denitrificans* is a soluble periplasmic enzyme which catalyzes the oxidation of methylamine to formaldehyde plus ammonia. The natural electron acceptor for this redox enzyme is a type I copper protein, amicyanin (Husain & Davidson, 1985). When assayed in vitro, mediators such as phenazine ethosulfate (PES)[1] are routinely used. Methylamine dehydrogenase possesses an $\alpha_2\beta_2$ structure and subunit molecular weights of 46 700 and 15 500 (Husain & Davidson, 1987). Each small subunit contains a covalently bound quinonoid cofactor. This enzyme is a member of a newly characterized family of bacterial and eukaryotic oxidoreductases which are referred to as quinoproteins [reviewed by Duine et al. (1987) and Duine and Jongejan (1989)]. Most bacterial quinoproteins, such as methanol (Duine & Frank, 1979) and glucose (Duine et al., 1979) dehydrogenases, possess a noncovalently associated *o*-quinone cofactor named pyrroloquinoline quinone (PQQ) (Salisbury et al., 1979). Certain eukaryotic amine oxidases are now also believed to possess novel quinone species at their active sites. For bovine plasma amine oxidase, it has been suggested that the covalently bound redox center is 6-hydroxydopa which may exist either as an *o*-quinone or as a *p*-quinone (Janes et al., 1990). For mammalian lysyl oxidase, the precise nature of the covalent cofactor is uncertain, but data suggest a covalently bound *o*-quinone such as PQQ is present (van der Meer & Duine, 1986; Williamson et al., 1986; Gacheru et al., 1989). Methylamine dehydrogenase is atypical of bacterial quinoproteins and similar to eukaryotic quinoproteins in that it possesses a covalently bound *o*-quinone at its active site. This cofactor was previously identified as PQQ (van der Meer et al., 1987). Recent data, however, suggest that the cofactor is not PQQ but an indole–quinone species which is structurally quite similar to PQQ and which is derived from two tryptophan residues of the structural protein.[2]

Many of the physical, kinetic, and redox properties of this enzyme have been previously characterized in this laboratory (Davidson & Neher, 1987; Husain & Davidson, 1987; Husain et al., 1987; Gray et al., 1988; Chen et al., 1988; Davidson, 1989; Davidson & Kumar, 1990; Davidson et al., 1990; Kumar & Davidson, 1990). During our studies of the mechanism of this enzyme, we have encountered an unusual type of inhibition caused by cyclopropylamine. Cyclopropylamines have been shown to be mechanism-based inhibitors of a variety of redox enzymes including cytochrome P-450 (Hanzlik & Tullman, 1982) and flavin-dependent oxidoreductases (Paech et al., 1980). Our data suggest a novel mechanism of inhibition by cyclopropylamine of this quinoprotein. These results further suggest an intimate role for the "non-catalytic" α subunit of this enzyme in establishing the functional environment at the active site of this enzyme.

Experimental Procedures

Methylamine, 2,6-dichlorophenolindolphenol (DCIP), PES, and cyclopropylamine were obtained from Sigma. Methylamine dehydrogenase and amicyanin were purified from *P. denitrificans* (ATCC 13543) as described previously (Husain & Davidson, 1986, 1987). Protein concentrations were calculated from previously determined extinction coefficients (Husain et al., 1987; Husain & Davidson, 1986). The reduced and semiquinone forms of methylamine dehydrogenase were generated either by reaction of the oxidized enzyme with sodium dithionite (Husain et al., 1987) or by reaction with methylamine under appropriate conditions (Davidson et al., 1990).

Methylamine dehydrogenase activity was measured spectrophotometrically with a dye-linked assay in which the oxidation of methylamine was coupled to a change in the absorbance of a redox-sensitive dye, DCIP (Davidson, 1989).

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[1] Abbreviations: PQQ, pyrroloquinoline quinone; PES, phenazine ethosulfate; DCIP, 2,6-dichlorophenolindophenol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

[2] L. Chen, F. S. Mathews, and V. L. Davidson, unpublished results.

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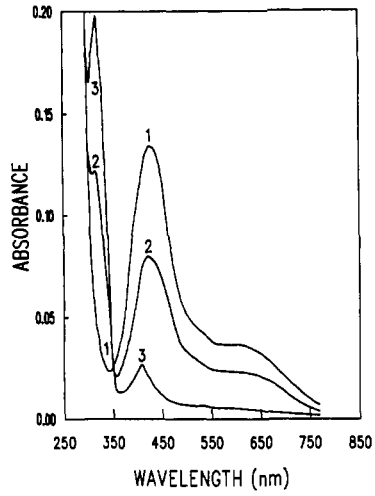

FIGURE 1: Spectral changes caused by cyclopropylamine addition to methylamine dehydrogenase. Oxidized methylamine dehydrogenase (4.6 μM) was present in 50 mM potassium phosphate, pH 7.5. Spectra were recorded before any additions (1), after addition of 4.6 μM cyclopropylamine (2), and after a second addition of 4.6 μM methylamine (3).

Absorbance spectra were recorded with a Kontron Uvicon 810 spectrophotometer. Excess reagents and noncovalently bound species were separated from the enzyme by passage over a small Ultrogel AcA 202 (IBF Biotechnics) desalting column. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed by the method of Laemmli and Favre (1973) except for the inclusion of 0.5 M urea in the resolving and stacking gel, and 4 M urea and 4% SDS in the final sample buffer. Electrophoretic transfer to nitrocellulose was performed with Bio-Rad reagents and equipment according to the instructions of the manufacturer. The procedure used for the detection of a functional protein-bound quinone species on blots by redox cycling was essentially as described by Paz et al. (1988) for assays done in solution. After transfer, the nitrocellulose sheet was soaked for 30 min in the dark in a solution of 2 M sodium glycinate, pH 10, with 0.01 M EDTA and 2 mg/mL nitroblue tetrazolium. Molecular weights were determined by comparison with Bio-Rad prestained low molecular weight standards. These standards were lysozyme (16 000), soybean trypsin inhibitor (21 500), carbonic anhydrase (33 000), ovalbumin (47 000), bovine serum albumin (84 000), and phosphorylase B (110 000).

Results

Reduction of Methylamine Dehydrogenase by Cyclopropylamine. Addition of cyclopropylamine to methylamine dehydrogenase caused changes in its visible absorption spectrum which indicated that the protein-bound cofactor was being reduced (Figure 1). A titration of these spectral changes caused by addition of cyclopropylamine indicated that complete reduction required a 2:1 molar ratio of reagent to enzyme. These spectral changes occurred rapidly and suggested that the cyclopropylamine was an effective substrate for the reductive half-reaction with this enzyme. No change in the absorption spectrum was observed on addition of cyclopropylamine to either the semiquinone or the reduced forms of the enzyme.

Kinetic Analysis of Cyclopropylamine Inhibition. Despite the fact that cyclopropylamine rapidly reduced methylamine dehydrogenase, it was not a substrate for the enzyme in steady-state assays of the enzyme with either the artificial electron acceptor PES or its natural electron acceptor amicyanin. Incubation of methylamine dehydrogenase with cyclopropylamine, in the presence of the reoxidant PES, caused time-dependent inactivation (Figure 2). A secondary plot of half-lives, determined from the initial slopes of each inactivation curve, against the reciprocal of cyclopropylamine concentration exhibited saturation behavior (Kitz & Wilson, 1962). The plot was linear and passed through the intercept at a positive value. This suggested that inactivation occurred by the simple model shown in eq 1. The parameters derived

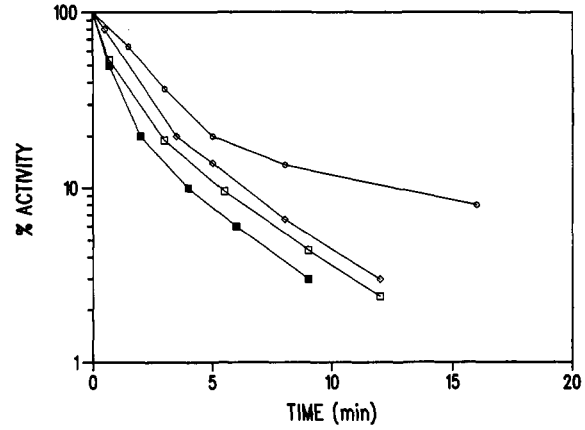

FIGURE 2: Time-dependent inactivation of methylamine dehydrogenase by cyclopropylamine in the presence of PES. Prior to being assayed, 1 μM methylamine dehydrogenase was incubated in 50 mM potassium phosphate, pH 7.5, in the presence of 4.8 mM PES and (○) 0.94, (◇) 1.6, (□) 3.1, and (■) 6.3 μM cyclopropylamine. At the indicated times, aliquots of the enzyme were removed and diluted 60-fold into the assay mixture.

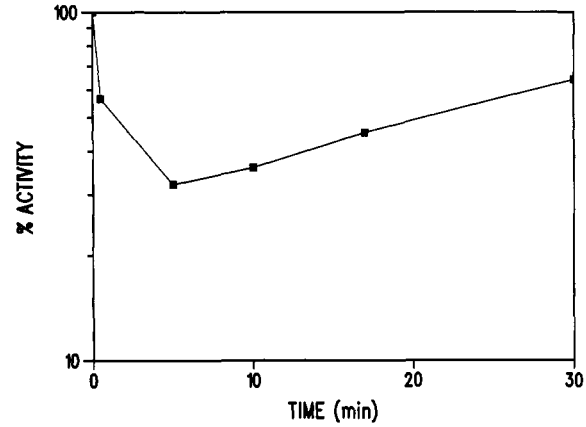

FIGURE 3: Time-dependent changes in methylamine dehydrogenase activity caused by incubation with cyclopropylamine in the absence of a reoxidant. Prior to being assayed, 1 μM methylamine dehydrogenase was incubated in 50 mM potassium phosphate, pH 7.5, with 12.5 μM cyclopropylamine. At the indicated times, aliquots of the enzyme were removed and diluted 60-fold into the assay mixture.

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} EI \xrightarrow{k} E_{inact} \quad (1)$$

from the secondary plots were K_I = 3.9 μM and K_{inact} = 1.7 min^{-1}. Incubation of the enzyme with cyclopropylamine in the presence of its physiological electron acceptor, amicyanin, also caused time-dependent inactivation. Incubation of methylamine dehydrogenase with cyclopropylamine in the absence of a reoxidant did not cause time-dependent inactivation (Figure 3). Instead a transient decrease in activity was observed at short incubation times with normal activity observed after longer incubation.

Demonstration of Irreversible Inactivation. To determine whether the inhibition of methylamine dehydrogenase was completely irreversible, the inactivated enzyme was passed over a small desalting column to remove any excess reagents and

Table I: Reoxidant-Dependent Irreversible Inactivation of Methylamine Dehydrogenase

present during incubation[a]	% act. after gel filtration
no additions	100
cyclopropylamine (0.3 mM)	100
cyclopropylamine (0.3 mM) + PES (0.1 mM)	1
cyclopropylamine (0.3 mM) + amicyanin (0.03 mM)	<1

[a] Methylamine dehydrogenase (5.8 μM) was incubated in 50 mM potassium phosphate, pH 7.5, for 2 h at room temperature with additions as indicated.

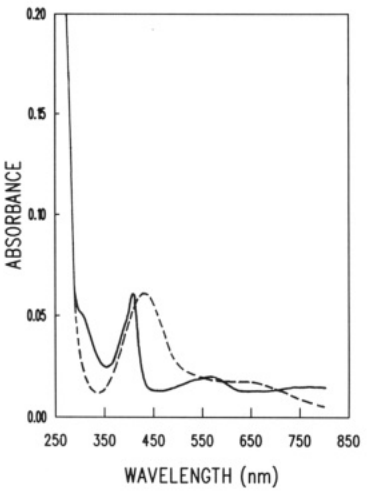

FIGURE 4: Absorption spectra of methylamine dehydrogenase before and after inactivation by cyclopropylamine. The solid line represents the spectrum of oxidized methylamine dehydrogenase. The dashed line is the spectrum of methylamine dehydrogenase which had been incubated with excess cyclopropylamine and PES for 2 h and then passed over a small desalting column to remove unbound species.

noncovalently bound species. The procedure was performed with enzyme that had been inactivated with cyclopropylamine alone, with cyclopropylamine and PES, and with cyclopropylamine and amicyanin (Table I). Enzyme which had been incubated in the absence of the reoxidant exhibited normal activity after passage over the column and exhibited an absorbance spectrum characteristic of the reduced form of the enzyme. Essentially no activity was observed after gel filtration of methylamine dehydrogenase which had been incubated with cyclopropylamine in the presence of either PES or amicyanin. These inactivated enzymes exhibited unusual absorption spectra (Figure 4) which were significantly different from those of oxidized, reduced, or semiquinone forms of the native enzyme.

Electrophoretic Analysis of Cyclopropylamine-Treated Enzyme. Methylamine dehydrogenase which had been incubated with cyclopropylamine was analyzed by SDS–PAGE. Electrophoretic analysis indicated that incubation of the enzyme with cyclopropylamine in the presence of either PES or amicyanin caused covalent cross-linking of the α and β subunits of this oligomeric enzyme (Figure 5). In this experiment, methylamine dehydrogenase was incubated with a 50-fold molar excess of cyclopropylamine. Where indicated, PES and amicyanin were present, respectively, at a 50-fold and 4-fold molar excess of methylamine dehydrogenase. Incubations were for 90 min at room temperature. Appearance of the band at a molecular weight of approximately 65 000 in lanes 4 and 5 in Figure 5A can only be explained by covalent cross-linking of the α and β subunits. No cross-linked species was observed in lane 3 for methylamine dehydrogenase which had been incubated with cyclopropylamine in the absence of a reoxidant. These samples were further examined by using a redox-cycling

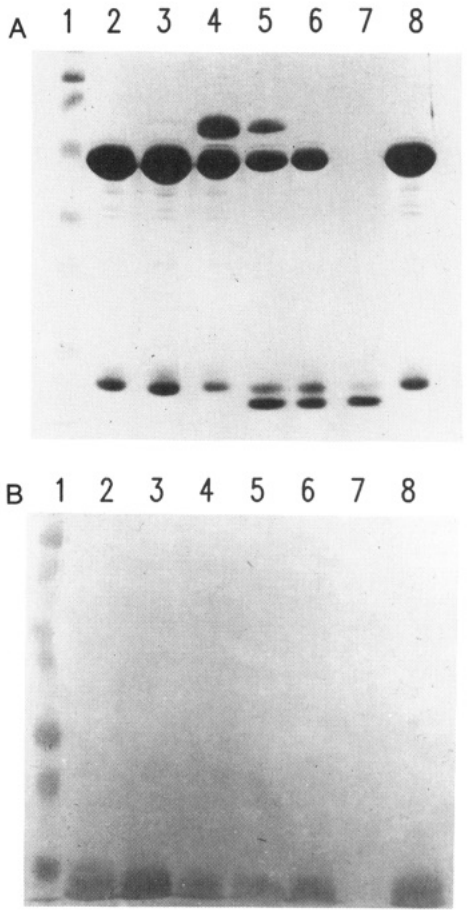

FIGURE 5: Electrophoretic analysis of cyclopropylamine-inactivated methylamine dehydrogenase. (A) Proteins were analyzed by SDS–PAGE on a 13.5% gel and stained with Coomassie Blue. Lanes contained (1) prestained molecular weight standards, (2) methylamine dehydrogenase (71 pmol), (3) methylamine dehydrogenase (71 pmol) which had been incubated with cyclopropylamine, (4) methylamine dehydrogenase (71 pmol) which had been incubated with cyclopropylamine and PES, (5) methylamine dehydrogenase (24 pmol) plus amicyanin (0.1 nmol) which had been incubated with cyclopropylamine, (6) methylamine dehydrogenase (24 pmol) which had been incubated with amicyanin (0.1 nmol) in the absence of cyclopropylamine, (7) amicyanin (0.1 nmol), and (8) methylamine dehydrogenase (71 pmol). (B) Redox cycling assay of proteins after electrophoretic transfer to nitrocellulose. The contents of this gel were identical with those in (A) except that in each case 3-fold more protein was used. The stain observed is due to deposition of formazan which is produced during redox cycling.

assay (Paz et al., 1988) to test for the presence of a functional quinone cofactor. The β subunits of methylamine dehydrogenase, present in all lanes except lane 7, showed a positive reaction, as expected (Figure 5B). The α–β cross-linked species present in lanes 4 and 5 showed no reaction at all, suggesting that the protein-bound quinone cofactor had been irreversibly modified during the cyclopropylamine-mediated cross-linking of subunits. From the intensities of the stained species in Figure 5A and in other experiments, it appeared that the maximum degree of cross-linking ever observed was approximately 50% of the total enzyme.

Discussion

The inhibition described here is unusual in a number of respects. Time-dependent inactivation of redox enzymes by cyclopropyl compounds is not uncommon. Some cytochrome P-450 dependent enzymes are inhibited by cyclopropylamines by what is believed to be a radical-based mechanism (Hanzlik & Tullman, 1982). For flavoprotein amine oxidases, it has

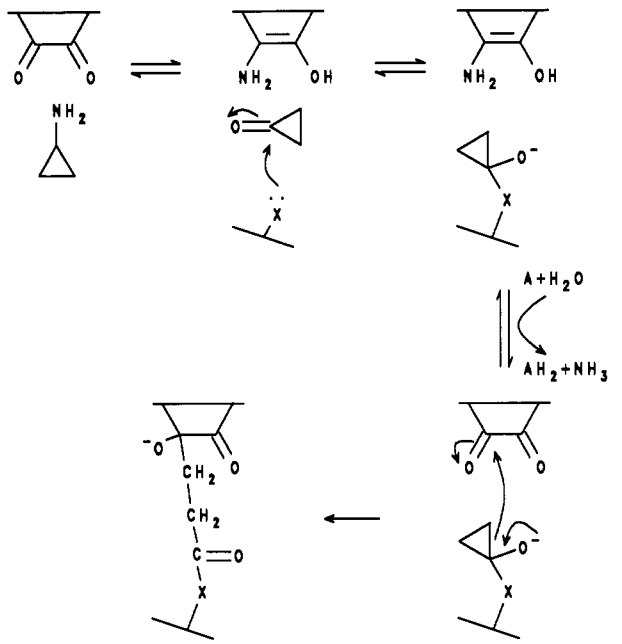

FIGURE 6: Proposed mechanism of cyclopropylamine inhibition of methylamine dehydrogenase.

been proposed that inhibition by cyclopropylamines occurs by a mechanism in which the amine is first oxidized by the redox cofactor and then reacts with an amino acid at the active site to form a covalent adduct (Paech et al., 1980). Similarly, with methylamine dehydrogenase, the reductive half-reaction appears to proceed normally. In this case, however, irreversible inhibition also required completion of the oxidative half-reaction. Furthermore, the observation that inactivation by cyclopropylamine caused covalent cross-linking of dissimilar subunits of an enzyme is unprecedented. The sum of the data obtained also suggest that the quinone cofactor has been modified in the inactivated enzyme. A rationale for these observations is that the product of the oxidative deamination of cyclopropylamine, cyclopropanone, initiates the inactivation process. A possible mechanism, which is consistent with our experimental results, is shown in Figure 6 and is described below.

Previous studies (Davidson, 1989) indicated that this enzyme obeyed a ping-pong mechanism with release of the aldehyde product prior to reaction with the reoxidant. The spectral changes caused by addition of cyclopropylamine (Figure 1) suggest that the reductive half-reaction of methylamine dehydrogenase proceeds normally. As such, a cyclopropanone product would be released at the active site of the enzyme. Such a species would be quite susceptible to nucleophilic attack. Reaction with an amino acid side chain near the cofactor could generate a covalently bound hemiketal species. Depending upon the nature of the amino acid side chain, X could be either oxygen, nitrogen, or sulfur which would yield, respectively, a hemiketal, aminohemiketal, or thiohemiketal intermediate. Such a species would be covalently, but reversibly, bound. As such, with time one would expect cyclopropanone to diffuse away from the active site. This would account for the rapid transient decrease, and subsequent slow increase of activity when the enzyme is incubated with cyclopropylamine in the absence of reoxidant (Figure 3). It is also consistent with the observation that the activity of enzyme incubated with cyclopropylamine in the absence of reoxidant is completely restored after gel filtration (Table I). Reoxidation of methylamine dehydrogenase will cause release of the second product, ammonia, from the cofactor and regenerate the original carbonyl function. When PES or amicyanin is present during incubation with cyclopropylamine, this will occur immediately after cyclopropanone release and hemiketal formation. It is proposed, under those conditions, that the protein-bound ketal species undergoes ring opening and formation of a transient reactive carbanion which would initiate a nucleophilic attack of the regenerated carbonyl carbon to form another covalent bond. The resultant inactive enzyme will thus be covalently cross-linked by the propyl group. The data suggest that the linkage will be between the quinone cofactor, which is covalently attached to the β subunit, and a nucleophilic side chain of an amino acid of the α subunit.

Another point of interest is the observation that at most 50% of the total enzyme subunits are cross-linked during inactivation. The spectral titration of methylamine dehydrogenase with cyclopropylamine indicated that both of the protein-bound cofactors became reduced and that each behaved equivalently in the reductive half-reaction. The 50% cross-linking may suggest that the two sites are not equivalent during the oxidative half-reaction. Clearly, cross-linking of one half of the $\alpha_2\beta_2$ enzyme prevents the reoxidant-dependent cross-linking of the second site, and this species is completely inactive.

The mechanism which was presented above presumes that some portion of the α subunit contributes to the environment which surrounds the active site. Such a suggestion is consistent with X-ray crystallographic data which have been obtained for this methylamine dehydrogenase[2] as well as for the enzyme from *Thiobacillus versutus* (Vellieux et al., 1989). These data indicated that the quinone cofactor is located in a narrow channel at an interface between the α and β subunits. These data are also consistent with observations that the resolved β subunit of methylamine dehydrogenase is completely inactive and exhibits an absorbance spectrum different than that of the holoenzyme (Husain & Davidson, 1987). An intriguing question in the study of redox enzymes is the nature of the precise role of "non-catalytic" subunits, those which do not possess a redox center and have no obvious role in catalysis. These data suggest that for methylamine dehydrogenase, the α subunit may play a critical role in establishing the functional environment at the active site of this enzyme.

Registry No. PES, 10510-77-7; methylamine dehydrogenase, 60496-14-2; cyclopropylamine, 765-30-0; amicyanin, 99490-02-5; PQQ, 72909-34-3.

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Comparison of Reduced and Oxidized Yeast Iso-1-cytochrome *c* Using Proton Paramagnetic Shifts†

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ABSTRACT: Dipolar paramagnetic shifts for protons of yeast iso-1-cytochrome *c* have been calculated by using an optimized **g**-tensor and the X-ray crystallographic coordinates of the reduced form of yeast iso-1-cytochrome *c* [Louie, G. V., & Brayer, G. D. (1990) *J. Mol. Biol. 214*, 527–555]. The calculated values are compared with the observed paramagnetic shift determined from over 450 nonequivalent protons that have been assigned in both oxidation states [Gao, Y., Boyd, J., Williams, R. J. P., & Pielak, G. J. (1990) *Biochemistry 29*, 6994–7003]. There is good agreement between the calculated and the experimental data with a few exceptions. This indicates that, overall, the solution structures must be very similar in both the reduced and oxidized states in solution as is the case in crystals. The differences between observed and calculated shift values for the molecule in solution are most readily explained by slight movement of the heme and certain changes in diamagnetic shift due to small rearrangements of a few residues and some considerable changes in a few hydrogen bonds. It is also known that small differences exist between the structures of the two oxidation states in crystals but the hydrogen-bond changes are not so easily observed there. Structural changes from nuclear magnetic resonance data are in reasonable agreement with those deduced from crystallography, but additional information is clearly available concerning changes in hydrogen bonding.

Cytochromes *c* exist in two physiologically important oxidation states. The reduced form is diamagnetic [Fe(II), low spin d^6, $S = 0$], while the oxidized form is paramagnetic [Fe(III), low spin d^5, $S = {}^1/_2$]. Takano and Dickerson (1981a,b) observed specific conformational changes between the X-ray crystal structures of reduced and oxidized tuna cytochrome *c*. These conformational changes are small (<1 Å) and occur in the vicinity of one of the propionic side chains of the heme. The work presented here follows earlier exploratory work based on paramagnetic nuclear magnetic resonance (NMR)[1] shifts for tuna cytochrome *c* (Williams et al., 1985). *Saccharomyces cerevisiae* iso-1-cytochrome *c* is used in the present study because this protein allows easy access to comparative mutational studies. A parallel study of horse heart cytochrome *c* has recently been reported by Feng et al. (1990). We have virtually identical data for horse heart cytochrome *c*, and although our analysis is different, it leads to similar conclusions (unpublished data).

The complete assignment of the C102T variant of yeast iso-1- and horse heart cytochromes *c* in both the oxidized and

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[1] Abbreviations: EPR, electron paramagnetic resonance; NOE, nuclear Overhauser enhancement; NMR, nuclear magnetic resonance; ppm, parts per million; rms, root mean square.

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