Although ligand binding in c-type cytochromes is not directly related to their physiological function, it has the potential to provide valuable information on protein stability and dynamics, particularly in the region of the methionine sixth heme ligand and the nearby peptide chain that has been implicated in electron transfer. Thus, we have measured the equilibrium and kinetics of binding of imidazole to eight mutants of *Rhodobacter capsulatus* cytochrome *c*₂ that differ in overall protein stability. We found that imidazole binding affinity varies 70-fold, but does not correlate with overall protein stability. Instead, each mutant exerts an effect at the local level, with the largest change due to mutant G95E (glycine substituted by glutamate), which shows 30-fold stronger binding compared with the wild-type protein. The kinetics of imidazole binding are monophasic and reach saturation at high ligand concentrations for all the mutants and wild-type protein, which is attributed to a rate-limiting conformational change leading to breakage of the iron–methionine bond and providing a binding site for imidazole. The mutants show as much as an 18-fold variation in the first-order rate constant for binding by cytochrome *c*₂, which is much more stable to guanidine denaturation than are the *c*₂-type cytochromes, and at least some of the difference in imidazole binding may be related to the disparity in overall protein stability. We have also shown that the imidazole binding properties of *R. capsulatus* and *R. sphaeroides* *c*₂-type cytochromes are almost identical. These properties, coupled with their amino acid sequence homology in the hinge region and overall structural homology of the native proteins, suggest that the *R. sphaeroides* cytochrome *c*₂-imidazole complex (3) is a reasonable model for that of *R. capsulatus* cytochrome *c*₂, for which there is no structure of the imidazole complex. Although there is no direct evidence for a role of the movement of the “hinge region” in electron transfer, this structural region has been implicated in binding to reaction partners (13). Moreover, protein dynamic processes have recently become a focus of protein chemistry as efforts to understand folding pathways and stability have expanded. Ligand binding by cytochrome *c*₂ provides an opportunity to investigate in real time an intrinsic property of a naturally occurring dynamic system.

We have now studied the equilibrium and kinetics of imidazole binding to a variety of *R. capsulatus* cytochrome *c*₂ mutants that differ in overall stability to guanidine denaturation. Fig. 1 presents a stereo view of the *R. sphaeroides* cytochrome *c*₂-imidazole complex and a similar view of *R. capsulatus* ferrocytochrome *c*₂, with the amino acid side chains that have been mutated shown in boldface (see below). For the purpose of the studies reported here, we are utilizing mutations at positions that are highly conserved among the c-type cytochromes and one mutation that is located in the hinge region. Thus, we are reporting mutations at Gly-34, Pro-35, Ile-57, Trp-67, Tyr-75, and Gly-95, which correspond to positions 29, 30, 52, 59, 67, and 79, respectively, in mitochondrial cytochrome *c*. Finally, we have inserted a valine between positions 10 and 11 in *R. capsulatus* cytochrome *c*₂. The amino acid at this position in the mitochondrial *c*₂-type cytochromes has been deleted in *R. capsulatus* cytochrome *c*₂. Comparison of the ligand binding properties of the mutants with those of the wild-type protein pro-

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‡ Present address: Dept. of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, MO 63110.

§ To whom correspondence should be addressed. Tel.: 520-621-7533; Fax: 520-621-9288; E-mail:cusano vict@u.arizona.edu.

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vide information on the role of overall protein stability in the movement of the hinge region and on more localized factors, which influence the immediate heme environment and electronic properties.

MATERIALS AND METHODS

Wild-type and mutant R. capsulatus c$_2$-type cytochromes were prepared as described previously (4). The spectral properties of the mutants including visible extinction coefficients were, within experimental error, the same as the wild-type protein. With some mutants, small changes in absorbance were observed in the near-IR (600–700 nm), where absorbance due to high spin species (620–640 nm) and the 695 nm charge transfer band are present. Unfortunately, this spectral region is complex, and the extinction coefficients are small and not easy to interpret. The G95E mutant shows larger spectral changes in the near-IR apparently connected with a substantial amount of high spin species present, although both the 695 nm species and the envelope are also changing. This makes it difficult to quantify.

The equilibrium binding of imidazole was studied by measuring the peak-to-trough difference in amplitude due to the increase in absorbance at 405 nm and the decrease in absorbance at 417 nm as described previously. The kinetics were followed at 417 nm. All experiments were carried out in 100 mM Tris cacodylate buffer, pH 7.0, supplemented with sodium chloride to maintain a constant ionic strength. All solutions contained 100 mM potassium ferricyanide to maintain the cytochrome c$_2$-imidazole complex, with the side chains of the heme ligands His-19 and Met-100 side chains, and imidazole shown in boldface. B, stereo view of the R. capsulatus cytochrome c$_2$ backbone structure, with the side chains of the heme ligands His-17 and Met-96 as well as mutation sites shown in boldface.

RESULTS AND DISCUSSION

Equilibrium Imidazole Binding—From our previous work comparing horse cytochrome c and R. capsulatus cytochrome c$_2$, it is possible that the difference in imidazole binding affinity may be related in part to overall protein stability (i.e., increased stability may result in decreased affinity for imidazole and vice versa). Therefore, we measured imidazole binding for eight R. capsulatus cytochrome c$_2$ mutants that differ in overall protein stability. Fig. 2A gives the difference absorbance spectra at different concentrations of imidazole that were obtained for mutant P35A (these results are typical of the wild-type protein and all mutants except G95E) (Fig. 3). The data were analyzed to obtain the overall apparent binding constant ($K_{a(app)}$), and the results are plotted in Fig. 2B. The values of $K_{a(app)}$ determined for the wild-type protein and all eight mutants are given in Table I. The overall affinities of the eight mutants for imidazole varied from 630 to 44,400 M$^{-1}$, which are all substantially larger than for horse cytochrome c (30 M$^{-1}$) (2). We found no correlation between mutant affinity for imidazole and protein stability. If $\Delta G^{\circ}_{u}$ is plotted against $\Delta G^{\circ}_{u}$, six of the mutants show a qualitative relationship between increasing affinity and decreasing overall stability (Fig. 4). These include P35A, P35T, G34S, W67Y, G95E, and I57N. The two mutants that are stabilized relative to the wild-type protein (Y75F and 10V11) clearly do not correlate. However, Y75F does have a decreased affinity for imidazole, which would be expected from a qualitative relationship to stability. Taken together, it would appear that global destabilization does not correlate with imidazole affinity, and as a consequence, local effects dominate. This is not unreasonable since the conformational change that leads to imidazole binding involves a segment of peptide chain (amino acids 88–102) that is not involved in regular secondary structure (which is what is measured by circular dichroism at 220 nm during guanidine denaturation) (3).

Average slope (3.18) obtained from the log plots for the mutants and wild-type protein (5). The differences in $\Delta G^{\circ}_{u}$, values for mutants relative to the wild-type protein were designated $\Delta G^{\circ}_{u}$. The convention used is that a positive value of $\Delta G$ reflects a decreased stability relative to the wild-type protein and that a negative value reflects an increased stability (see Ref. 6 for further discussion).

Overall protein stability was measured by guanidine denaturation followed by the change in the circular dichroism signal at 220 nm. The guanidine concentration at the midpoint of denaturation ($C_{u}$) was used to compare the mutants with the wild-type protein. The free energy of denaturation ($\Delta G^{\circ}_{u}$) was obtained by multiplying the $C_{u}$ values by the average slope (3.18) obtained from the log plots for the mutants and wild-type protein (5).
denaturation) and is not necessarily directly influenced by mutations distant from the hinge region.

Mutant G95E has the largest affinity of any of the mutants or the wild-type protein (30-fold more than the wild-type protein). Given its location in the proposed hinge region, this is not surprising in itself. However, G95E also has difference spectra that are strongly perturbed relative to the wild-type protein and the other mutants (Fig. 3). Thus, what appears to be a relatively benign mutation (because it is at a position that is not conserved among the c-type cytochromes) results in a significant perturbation of the heme electronic structure. As glycine is the only amino acid without a side chain, it may in some cases adopt dihedral angles not allowed by the others and thus would be particularly sensitive to mutation. However, Gly-95 has normal \(\phi-\psi\) angles (9), suggesting that mutation at this position will not have direct structural effects on the peptide backbone. It is more likely that the glutamate in G95E interacts unfavorably with the front heme propionate, forcing the peptide chain in the hinge region into a less stable conformation (see below).

The binding of imidazole to cytochromes occurs in two steps, a protein rearrangement involving breakage of the iron-methionine bond followed by binding of imidazole to the iron (Equation 1). Moreover, the alkaline \(pK_{695}\) supposedly measures the strength of the iron-methionine bond. Two of the mutants, W67Y and I57N, have a significantly lower \(pK_{695}\) than the wild-type protein and thus may show differences in imidazole binding at pH values above 7 but below pH 10, where lysines and tyrosines begin to deprotonate. Therefore, the affinities of wild-type cytochrome \(c_2\) and mutant W67Y for imidazole were determined over the pH range 7–10 at a constant ionic strength (500 mM). For the wild-type protein, the difference absorbance spectrum was altered at pH 10, but the affinity only decreased by a factor of 2 (\(K_{a\text{app}} = 710 \pm 30 \text{ M}^{-1}\)) between pH 7 and 10. In contrast, for mutant W67Y, the onset of the spectral change was observed at pH 9, and the apparent affinity had changed dramatically (from 630 \(\pm 20 \text{ M}^{-1}\) at pH 7 to 40 \(\pm 0.6 \text{ M}^{-1}\) at pH 10). These observations are consistent with the 1 pH unit shift to a lower value in the \(pK\) for disappearance of the 695 nm absorbance band in the W67Y mutant. Thus, at high pH, there is a conformational change that results in a 16-fold decrease of the global affinity for imidazole, which qualitatively is related to the alkaline \(pK_{695}\) in W67Y. However, we expected the affinity for imidazole to increase at high pH, corresponding to the loss of the methionine ligand and the increased availability of the sixth coordination position. If the methionine is replaced by another strong field ligand from the protein as expected from the literature (7), then the affinity for that ligand must be stronger than that for imidazole. In any case, there is no apparent correlation between imidazole affinity at pH 7 and the \(pK_{695}\) among the mutants studied (see Table 1). Thus, structural or dynamic changes resulting in perturbation of the 695 nm band are not directly related to the dynamic equilibrium in the hinge region. Consistent with the results with cytochrome \(c_2\), it was shown (8) that there is no pH dependence of the \(K_{a\text{app}}\) of horse ferricytochrome \(c\) for imidazole between pH 6 and 8, i.e. below the \(pK_{695}\).

The lack of correlation between imidazole binding affinity and either overall protein stability or the \(pK\) suggests that each mutation has local effects that do not directly reflect the more global properties. Alternatively, \(K_{a\text{app}} = K_a/1 + K_a K_b/(1 + K_b)\) may need to be resolved into the two equilibria (described by Equation 1) and the corresponding kinetic parameters to accurately interpret the impact of mutation, i.e. the two
equilibria could be affected differently by mutation. Therefore, we have analyzed the stopped-flow kinetics of the reaction of imidazole with the wild-type and mutant cytochromes to determine whether there is any correlation between the kinetic rate constants and either of the global stability measurements, $C_M$ and $pK_{695}$.

Stopped-flow Kinetics—The kinetics of the reaction of the mutants of *R. capsulatus* cytochrome *c*$_2$ with imidazole at pH 7 are monophasic at all ligand concentrations. A typical profile of the kinetics of the binding of imidazole (25 mM) to mutant P35A are monophasic at all ligand concentrations. A typical profile of the kinetics of the binding of imidazole (25 mM) to mutant P35A is shown in Fig. 5. Plots of $k_{\text{obs}}$ versus ligand concentration are nonlinear and demonstrate a change in the rate-limiting step at high ligand concentrations as shown in Fig. 6. Analysis of the kinetic data can be according either to the steady-state approximation (2) or to the explicit solution. From the steady-state analysis, $k_{12}$, $k_{23}$, and the ratio $k_{23}/k_{21}$ can be obtained and are given in Table II. The explicit solution (termed the transient kinetic analysis) yields the same parameters, and the resulting rate constants are also given in Table II. The agreement between the two methods was excellent, indicating that the steady-state approximation is appropriate for these reactions.

The limiting rate constant $k_{12}$ measures the breakage of the iron–methionine bond and rearrangement to the open form of the protein that is primed to bind imidazole. As was the case with the imidazole equilibrium affinity constant, there is no apparent correlation between the rate constant for rearrangement and overall protein stability or $pK_{695}$. For example, mutant 10V11 rearranges with a larger rate constant than the wild-type protein, yet it is substantially more stable to denaturation. Mutant G95E rearranges more rapidly than any other mutant, but is only moderately less stable, and mutant W67Y rearranges with a smaller rate constant than the wild-type protein, although it is much less stable than the others. Qualitatively, I57N, P35T, P35A, and G34S behave more or less as expected from their decreased stabilities (Table I) and have substantially increased rate constants for opening ($k_{12}$) (Table II). Mutant Y75F has a significantly higher $pK_{695}$ and overall stability than the wild-type protein and rearranges only slightly more slowly. Thus, we must conclude that local effects control the kinetics of rearrangement ($k_{12}$) as well as the overall equilibrium for imidazole binding as discussed above.

The second rate constant obtained from data analysis ($k_{23}$) measures the breakage of the imidazole–iron bond (or a rate-limiting step leading to bond breakage). The value of $k_{23}$ varies <3-fold among the mutants studied, and it is not perturbed significantly relative to the wild-type protein. This suggests that the electronic properties of the heme in the mutants are very similar to those of the wild-type protein and that stabilization of the imidazole–iron complex is not significantly altered by the mutations studied. These results are consistent with the presence of a stabilizing hydrogen bond with the imidazole in all of the mutants as well as in the wild-type protein.

The steady-state and transient kinetic approaches do not provide precise values of $k_{21}$ and $k_{23}$, but rather the ratio $k_{23}/k_{21}$. The individual rate constants are not known with precision as a consequence of the fact that a range of values for both $k_{23}$ and $k_{21}$ is found that yields generally similar least-squares errors from the fits as long as the ratio $k_{23}/k_{21}$ is similar to the value given in Table II. This is unfortunate in that it prohibits calculation of $K_1$ and $K_2$ with any precision. However, we know that the amount of the open form (presumably high spin heme) in the wild-type protein must be small at equilibrium. A variety of physical-chemical measurements including NMR, redox titrations, and the near-IR absorption spectra between 600 and 700 nm are all consistent with a predominantly low spin heme protein. Nevertheless, it is clear that $k_{23}/k_{21}$ can vary over a significant range for the mutants (0.7–4.4) (Table II), suggesting large changes in $k_{23}$ or $k_{21}$ or both. This leaves open the possibility that detectable amounts of the open form could be present in the mutants. As shown in Fig. 7, for P35T, 10V11, and I57N, there appear to be small increases in absorbance in the 600–650 nm region, where the high spin heme would be expected to absorb. This suggests $K_2$ values significantly higher than for the wild-type protein. Unfortunately, in the absence of a well developed understanding of small absorption bands in the 600–650 nm region of low spin c-type cytochromes, a quantitative analysis is not possible. Moreover, as shown in Fig. 3, the G95E difference spectrum is substantially altered relative to the wild-type protein and the other mutants, demonstrating both a significant perturbation

### Table I

| Cytochrome       | $K_{\text{app}}$ (μM) | $\Delta G_{K_2}$ (cal/mol) | $pK_{695}$ | $\Delta G_{K_2}$ (cal/mol) | $C_M$ (μM) | $\Delta G_{\text{u}}$ (cal/mol) |
|------------------|-----------------------|-----------------------------|------------|-----------------------------|------------|-----------------------------|
| *R. capsulatus* c$_2$ | 1440 ± 40             | -0.1                        | 8.9        | 0.0                         | 1.54       | +2.0                        |
| Mutant P35A      | 1660 ± 50             | +0.3                        | 8.9        | +0.3                        | 0.90       | +2.0                        |
| Mutant P35T      | 915 ± 20              | +0.2                        | 8.7        | +0.3                        | 0.92       | +2.0                        |
| Mutant Y75F      | 1010 ± 20             | 0.0                         | 10.7       | -2.4                        | 1.76       | -0.7                        |
| Mutant W67Y      | 630 ± 20              | +0.5                        | 8.0        | +1.2                        | 0.54       | +2.2                        |
| Mutant I57N      | 4800 ± 90             | -0.7                        | 7.9        | +1.3                        | 1.0        | +1.7                        |
| Mutant 10V11     | 2750 ± 20             | -0.4                        | 9.2        | -0.4                        | 2.0        | -1.5                        |
| Mutant G34S      | 2120 ± 20             | -0.2                        | 8.6        | +0.4                        | 0.86       | +2.2                        |
| Mutant G95E      | 44400 ± 800           | -2.0                        | ND*        | ND*                         | ND*        | +0.9                        |

* ND, not determined.
of the heme electronic structure and an increase in the amount of high spin heme as compared with the other mutants and the wild-type protein (Fig. 7).

Proline at position 35 or its structurally equivalent position is absolutely conserved in cytochromes $c$ and $c_2$. This is presumably a consequence of the Pro-35–His-17 hydrogen bond. NMR studies on the $R$. capsulatus wild-type protein and P35A suggest that although the overall structures of the two proteins are generally similar, there are significant dynamic differences (10–12). Notable are the increased flip rates of Phe-51 and Tyr-53, which are in the structural region of Pro-35 in the folded protein, and the increased exchange rates for N1H of His-17 and NH of Gly-34 in the P35A mutant. NH exchange rates also increase for residues 16, 18, 20, 37, 43, 45–46, and 58, all structurally proximal to the site of mutation. Based on both the chemical shift data for the oxidized protein$^2$ and exchange rates, there is no evidence from the NMR studies for perturbation in the previously identified hinge region (amino acids 88–102). The apparent imidazole affinities for P35A and P35T are only slightly different as compared with each other and to the wild-type protein. Moreover, $k_{32}$ and $k_{32}$ are essentially identical for P35A and P35T, and $k_{32}$ is not significantly different compared with the wild-type protein. However, $k_{12}$ is substantially increased in the two proline mutants (3-fold) relative to the wild-type protein, suggesting that the hinge region is moving between the open and closed forms at a substantially increased rate compared with the wild-type protein, consistent with destabilization of the hinge propagated from the mutation site.

Glycine at position 34 or its structurally equivalent position in the $c$-type cytochromes has been conserved throughout evolution. Adjacent to proline 35, glycine 34 is generally believed to be critical to the maintenance of the protein structure that positions and orients the ligated histidine. The hydrogen bond between the Gly-34 amide NH and the Cys-16 carbonyl may be an important factor relating to this functional role. Based on the NMR chemical shifts,$^2$ oxidized G34S is perturbed in a similar fashion to that observed for P35A relative to the wild-type protein. However, there are chemical shifts in region 95–105 in G34S that are not observed in P35A. Thus, it appears that perturbations at positions 34 and 35, resulting from mutation, can be propagated to the hinge region, leading to generally similar increases in $k_{12}$. Given the differences in NMR properties (G34S versus P35A) discussed above and the spectral perturbations given in Fig. 7, it appears that the pathways or mechanisms for propagation are somewhat different, but yield generally similar outcomes.

Trp-67 is highly conserved among the $c$-type cytochromes and provides a hydrogen bond between the tryptophan N1H and the rear propionate in the reduced state (9). Mutation of position 67 to tyrosine leads to a global destabilization of 2.2 kcal/mol (Table I). It appears that the tyrosine substitution still

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permits the propionate hydrogen bond (based upon a low resolution crystal structure). Moreover, the difference in free energy between tryptophan and tyrosine, when transferred from a hydrophobic medium to water, predicts a destabilization of 2.2 kcal/mol and accounts for the difference in guanidine stabilization of the oxidized form relative to the wild-type protein. Despite a net destabilization in W67Y, $k_{12}$ is decreased, suggesting a stabilization of the hinge region relative to the wild-type protein. Tyrosine has greater hydrogen bonding capability than tryptophan, and it is possible that it strengthens the hydrogen bond network involving Thr-94 and Tyr-75 (see below).

In the context of the mutants described up to this point, Y75F is unique in that the mutation leads to an overall stabilization of the oxidized form relative to the wild-type protein. However, this is counterintuitive since the more hydrophobic side chain would be expected to destabilize the oxidized form of the protein due to the uncompensated positive charge on the heme. In the reduced wild-type cytochrome, Tyr-75 is involved in a hydrogen bond network that includes Met-96, a bound water molecule, Thr-94, and the front heme propionate, which would be expected to be substantially altered by substitution of tyrosine by phenylalanine in the Y75F mutant (9). In yeast cytochrome c, the structure of the Y67F equivalent of the Y75F mutant of R. capsulatus shows that there is an additional water molecule present that participates in the hydrogen bond network and takes the place of the Tyr-67 hydroxyl (13). In view of the fact that Tyr-75 interacts with two hinge residues (Thr-94 and Met-96) and that mutant Y75F has similar imidazole binding characteristics as the wild-type protein, it is reasonable to expect maintenance of the hydrogen bond network in R. capsulatus due to insertion of a water molecule in Y75F by analogy to yeast. Despite the fact that the $pK_{695}$ is substantially increased, the effects of the mutation on imidazole binding are relatively small, with a decrease in the $k_{22}/k_{21}$ ratio that is in large part compensated for by a decrease in $k_{21}$. It appears reasonable that in terms of the increase in overall stability, the dissociation rate constant ($k_{32}$) is also decreased.

Mutant 10V11 involves an insertion between the N-terminal helix and the heme-binding site. There is no available structural information, but the insertion could cause a small bulge or a more dramatic rotation in the N-terminal helix, altering the interactions between the N-terminal helix and adjacent structural elements. Major rearrangements on the C-terminal side of the insertion are not likely because of the thioether bond between Cys-13 and the heme. Any interactions that are disrupted by the 10V11 insertion are apparently compensated for by new interactions with the hydrophobic side chain. However, the site of the insertion is spatially very close to the C terminus of the hinge region, and Lys-102 apparently forms a salt bridge (3.8 Å) with Glu-9. If the helix is rotated as a result of mutation, then this salt bridge could be disrupted. Lys-12 is also apparently salt-bridged to Glu-9 (3.2 Å), and movement of Lys-12 as a result of the insertion could disrupt this interaction as well. The increased affinity for imidazole by 10V11 is the consequence of an increase in $k_{12}$ and a decrease in $k_{21}$, with the ratio $k_{22}/k_{21}$ the same as the wild-type protein. This suggests that the Lys-102 salt bridge with Glu-9 was weakened by mutation, resulting in increased movement of the hinge region.

Position 57 in R. capsulatus cytochrome c$_2$ is structurally homologous to position 52 in yeast cytochrome c, which is an asparagine. The N52I mutation in yeast cytochrome c results in substantial stabilization of the protein. Thus, the substitution of isoleucine 57 with asparagine in R. capsulatus cytochrome c$_2$ results in destabilization, consistent with the yeast cytochrome c results, and points to the importance of a large hydrophobic

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**TABLE II**

|        | $k_{12}$ | $k_{22}$ | $k_{22}/k_{21}$ |
|--------|---------|---------|-----------------|
|        | SS      | TK      | SS              | TK              | SS      | TK              |
| Wild-type c$_2$ | 27.4 ± 0.8 | 26.8 | 0.063 ± 0.003 | 0.061 | 3.2 ± 0.2 | 3.4 |
| Mutant P35A | 88 ± 6 | 87.6 | 0.063 ± 0.003 | 0.061 | 1.20 ± 0.09 | 1.2 |
| Mutant P35T | 80 ± 10 | 82 | 0.055 ± 0.003 | 0.051 | 0.63 ± 0.05 | 0.7 |
| Mutant Y75F | 22 ± 1 | 22.2 | 0.038 ± 0.001 | 0.037 | 1.70 ± 0.09 | 1.8 |
| Mutant W67Y | 15.9 ± 0.3 | 15.7 | 0.106 ± 0.004 | 0.106 | 4.2 ± 0.2 | 4.4 |
| Mutant I57N | 143 ± 6 | 140 | 0.080 ± 0.004 | 0.074 | 2.7 ± 0.1 | 2.9 |
| Mutant 10V11 | 42.8 ± 0.8 | 41 | 0.043 ± 0.001 | 0.045 | 2.76 ± 0.07 | 3.3 |
| Mutant G34S | 83 ± 5 | 84 | 0.056 ± 0.004 | 0.053 | 1.4 ± 0.1 | 1.4 |
| Mutant G95E | 280 ± 25 | 255 | 0.095 ± 0.001 | 0.095 | 15 ± 2 | 20 |

* SS, steady-state approach; TK, transient kinetic approach.

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3 M. M. Benning and H. M. Holden, unpublished data.
side chain at this position. The side chain of mutant I57N is probably hydrogen-bonded to the rear heme propionate and the network including Tyr-75, Thr-94, and the internal water, just as Asn-52 is in yeast (13), but our imidazole binding results suggest that the interaction of Thr-94 with the hydrogen bond network may be disrupted as a result of the mutation. The increase in imidazole affinity relative to the wild-type protein (3-fold) is accounted for by a substantial increase in $k_{12}$, which would be consistent with a loss of the Thr-94 hydrogen bond with the front heme propionate and the internal water. This deserves further study.

The mutation of Gly-95 to Glu has profound effects on imidazole binding as well as on the spin state of the heme iron and overall protein stability. Clearly, there is a substantial shift in the equilibrium to the open form (Fig. 7), and the interconversion of the closed and open forms is much more rapid than in the wild-type protein. In contrast, $k_{32}$ is increased by $\sim 40\%$, suggesting that once the imidazole-G95E complex is formed, it is still stabilized by the hydrogen bonding process described for wild-type protein.\(^1\) The glycine at position 95 adopts normal dihedral angles, so this cannot be the reason for the decreased stability of G95E. However, the $\alpha$-carbon of Gly-95 is only 3.2 Å from the front propionate carboxyl group. The introduction of Glu at this position would clearly clash with the propionate both because of its size and due to its charge. It would thus force a rearrangement of the hinge or the propionate or both, resulting in a loss of hydrogen bonds or other favorable interactions.

**Conclusions**—We have shown that mutations in cytochrome $c_2$ can strongly influence the affinity for imidazole and the rate constant for formation of the open form ($k_{12}$). However, there are a number of limitations on the interpretation of the kinetic and equilibrium parameters measured. These include the fact that the perturbations are relatively small (70-fold in $K_a^{(app)}$ or 2.5 kcal/mol in energetic terms and 18-fold in $k_{12}$); there is no structural information available for the mutants beyond some NMR chemical shift data (i.e. there are no crystal or solution structures); and the rate constants $k_{21}$ and $k_{23}$ are not resolvable. Nevertheless, several general conclusions can be drawn. First, the imidazole affinity does not correlate with the stability of the mutants; rather, it appears to be influenced by local effects that presumably alter $K_1$ and/or $K_2$ differentially depending on the specific mutation. Second, $k_{12}$ is relatively easily perturbed by mutation both proximal and distal to the hinge region, suggesting that the changes in dynamic properties can be propagated throughout the folded molecule. Third, there is no obvious relationship between the $pK_a^{G95}$ and imidazole binding kinetics at pH 7. Thus, under these conditions, it appears that breakage of the iron–methionyl sulfur coordination bond is not rate-limiting. Moreover, the open and closed forms of cytochrome $c_2$ must be in a rapid equilibrium since the kinetics are homogeneous (single pseudo first-order process) irrespective of $K_1$ (Fig. 7). Fourth, $k_{32}$ is relatively insensitive to mutations as it varies only slightly among the forms of cytochrome $c_2$ studied (2.8-fold). This suggests that the stabilizing interactions, presumably a hydrogen bond involving the bound imidazole, an internal water, and the Phe-102 backbone carbonyl, are not altered significantly by the mutations studied.

It is clear that imidazole binding (equilibrium and kinetics) coupled with site-directed mutagenesis provides a means to study the role of adjacent structural domains and specific amino acid side chains on the dynamic properties of the hinge region. As more detailed structural information on the various cytochrome $c_2$ mutants becomes available, it should be possible to better understand, in molecular terms, the interactions between domains within cytochrome $c_2$ as well as the molecular dynamics that define its structure and function.

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