Synergy in Protein Engineering
MUTAGENIC MANIPULATION OF PROTEIN STRUCTURE TO SIMPLIFY SEMISYNTHESIS*

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Semisynthesis is a chemical technique of protein engineering that provides a valuable complement to directed mutagenesis. It is the method of choice when the structural modification requires, for example, a non-coded amino acid. The process involves specific and limited protein fragmentation, structural manipulation of the target sequence, and subsequent religation of fragments to give the mutant holoprotein. We suggested and demonstrated that mutagenesis and semisynthesis could be used synergistically to achieve protein engineering goals otherwise unobtainable, if mutagenesis was used to shuffle methionine residues in the yeast cytochrome c sequence (Wallace, C. J. A., Guillemette, J. G., Hibiya, Y., and Smith, M. (1991) J. Biol. Chem. 266, 21355–21357). These residues can not only be sites of specific cleavage by CNBr but also of spontaneous peptide bond synthesis between fragments in noncovalent complexes, which greatly facilitates the semisynthetic process. We have now used an informed “methionine scan” of the protein sequence to discover other useful sites and to characterize the factors that promote this extraordinary and convenient autocatalytic religation. Of eight sites canvassed, in a wide range of settings, five efficiently provoked peptide bond synthesis. The principal factor determining efficiency seems to be the hydrophobicity of the religation site. The mutants created have also provided some new insights on structure-function relationships in the cytochrome.

The ability to engineer protein structure became generally available with the development of site-directed mutagenesis (1) by virtue of its combination of simple chemistry and genetic manipulation, and the exploitation of the replicative fidelity and efficiency of living organisms. Before this breakthrough, structural manipulation had been undertaken at the level of the protein, rather than the gene that encodes it, by total synthesis or semisynthesis. These chemical methods suffered a number of limitations, sufficient enough that their use has been relatively restricted, although of late methods to avoid many of the difficulties have evolved. For a full discussion of these developments, see Kent (2), Muir and Kent (3), Offord (4, 5), and Wallace (6, 7).

The continuing desirability of facile chemical methods stems from their capabilities in areas where molecular biological approaches are themselves limited. These include the stable expression of partial, toxic, or grossly deformed sequences (8), the introduction of unnatural side chain structures (9), the insertion of labeled natural residues at specific sites in a protein (10), or the creation of dendrimeric or pennant structures (11).

The most troublesome aspect of chemical methods has been the inefficiency of the final peptide ligation step in fragment-condensation synthesis and semisynthesis, and hence we and others have sought ways to improve it (7). Our focus has been on processes that we term autocatalytic fragment religation (AFR).† There are many cases of complexation of peptides derived from limited proteolysis reactions to give structures with native-like conformation and, often, function. While the retained functionality itself implies that such complexes adopt the native fold, in some cases the implication has been verified by x-ray crystallography and spectroscopic techniques (12). Most studies have examined two-fragment noncovalent complexes (13), but examples with three (14) or four fragments (15) are known. One remarkable aspect of these complexes is that the close proximity of the termini at the breakpoints necessitated by the native fold can catalyze the spontaneous reformulation of the missing peptide bond(s), in thermodynamically favorable circumstances. Normally, reaction between ionized carboxylate and ammonium groups is disfavored, but in the case of CNBr fragments, where the C-terminal residue is homoserine lactone, it is not. Hence, for some proteins, incubation under renaturing conditions of a mixture of the cleavage products not only leads to complexation, but peptide bond formation too. Aminolysis of the lactone is a slow process (16), so that peptide bond formation between noncomplexing fragments is not detectable, but the proximity effect in a complex gives a many thousandfold rate enhancement, and makes it competitive with hydrolysis. In other words, complex formation effectively converts an intermolecular reaction into an entropically favorable intramolecular reaction. An analogous, but more complicated, process has been developed by us to utilize complexes of tryptic fragments (17).

Cytochrome c was the protein in which this type of spontaneous peptide bond formation was first observed (18, 19) and the religation of chemically manipulated or totally synthetic CNBr fragments 1–65 or 66–104 with the native partner has been the source of over 100 informative engineered analogs of the cytochrome (6), based overwhelmingly on the horse structure. The relative infrequency of methionine residues, in cyto-

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†‡ The abbreviations used are: AFR, autocatalytic fragment religation; MOPS, 3-(N-morpholino)propanesulfonic acid; TMPD, N,N,N,N′-tetramethyl-p-phenylenediamine dihydrochloride; HPLC, high performance liquid chromatography.
Synergistic Use of Directed Mutagenesis and Semisynthesis

chromes c as in other proteins, is both an aid to limited fragmentation and a hindrance to flexibility in cleavage sites, so much so that interesting residues can be inaccessible for semisynthesis. In addition, it is clear from the examples where spontaneous religation does not occur that subtle structural reasons dictate that some natural locations of methionine residues are not conducive to peptide bond formation. Thus, it occurred to us that we might use mutagenesis of the native protein to manipulate the distribution of noncrucial methionine residues and so prepare it for a subsequent convenient and facile semisynthesis, and that this synergy would permit engineering of the cytochrome in ways neither technique alone would permit. Additional benefits would arise from the use of the yeast sequence: it has become the paradigm for structure-function studies for cytochrome c; and facile semisynthesis, and that this synergy would permit protein to manipulate the distribution of noncrucial methionine residues and so prepare it for a subsequent convenient and facile semisynthesis.

The first trial of this strategy involved replacement of a pre-existing methionine in the yeast sequence. Located at position 64, this residue is susceptible to the CNBr cleavage reaction, but the resulting two-fragment complex does not undergo spontaneous religation. Replacing Met\(^{64}\) with Leu, and Ser\(^{65}\) by Met, as in the horse protein, led to AFR with a comparable efficiency, and permitted the creation of a semisynthetic analog, a chimera of the yeast and horse sequences, of *Saccharomyces* cytochrome c (20, 21). At the same time a similar approach was used to make various CNBr fragments of Barnase for complementation studies (22), but religation was not noted.\(^3\)

There are a number of reasons for extending that initial study. Choosing to insert the new methionine at the position corresponding to that which works in horse maximizes the chances of success, and proves the principle, but does not provide new information on the structural circumstances in which the spontaneous religation is promoted or repressed. This can only be had by canvassing a number of novel sites representative of varied conformational contexts, to show this is a general, rather than specific phenomenon, and hopefully to obtain clear predictive data on the relationship between site location and efficiency. We also wanted a range of evolutionary conservation to explore the importance of this factor in site selection. Finally, we wished to make functioning AFR systems that would open up new regions of the sequence for engineering by semisynthesis.

We have therefore developed six novel mutants of the yeast protein that provide a partial “methionine scan” of the sequence between residues 25 and 75 and subjected them to physicochemical and biological characterization as well as determining the capacity of two-fragment complexes derived from them to undergo the desired spontaneous resynthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**

All yeast iso-1-cytochrome c mutants were prepared by using recombinant techniques as described below. Hexokinase, cyanogen bromide, 3-[N-morpholino]propanesulfonic acid (MOPS), N\(_2\)N,N\(_2\)N\(_2\)-tetramethyl-

\(-p\)-phenylendiamine dihidrochloride (TMSP), and polyoxyethylene sorbitan monoooleate (Tween 80) were obtained from Sigma. ATP was from Boehringer Mannheim. Other chemicals were obtained from Baker (Toronto, Canada), BDH (Toronto, Canada), Fisher, or Fluka (Ronkonkoma, NY).

**Methods**

**Mutant Preparation**

Amino acid residues at sites selected for CNBr cleavage and homoserine lactate-mediated AFR in yeast iso-1-cytochrome c were replaced by methionine. Mutant cytochromes c were generated using the site-directed mutagenesis method (21, 22). Mutation was carried out on an earlier modified version of the naturally occurring yeast iso-1-cytochrome c gene in which the codon for Cys at position 102 was replaced with that of Thr; the resulting product does not have problems with dimerization and autoreduction during purification that mark the wild-type. This gene shall be called CYC1. The phagemid carrying the CYC1 gene (pING 4) bears all the elements necessary for replication in yeast and bacteria and may be converted into a single-stranded form of DNA for site-directed mutagenesis and nucleotide sequencing (23). Met\(^{64}\) was replaced with Leu in this series of mutants in order to limit the number of side cleavages; since this is a naturally variable site, the change is not considered to be significant. Met\(^{65}\) is an essential residue and was not modified. This gene will be referred to as [Leu64] CYC1.

Single mutation variants of the [Leu64] CYC1 gene product were subsequently produced. Pro\(^{25}\), Val\(^{28}\), Ile\(^{35}\), Lys\(^{55}\), Leu\(^{68}\), and Ile\(^{75}\) were chosen to be replaced with Met. Throughout this report the vertebrate cytochrome c nomenclature is used to conform with general practice. *Saccharomyces cerevisiae* has an N-terminal extension of 5 residues, and hence numbering of this sequence begins at residue -5. The pING 4 phagemid carrying the mutant cytochrome c gene was introduced into a strain of yeast deficient in both types of cytochrome c, GM3C-2 (24). Each variant form of yeast iso-1-cytochrome c was expressed in GM3C-2 cells and the recombinant protein purified as described previously (21).

**Molecular Modeling**

Modeling of the variants of yeast iso-1-cytochrome c was performed on an Iris Indigo (Silicon Graphics, Mountain View, CA), using the programs Insight II and Discover (Biosym Technologies, San Diego, CA). The x-ray crystal structure for the reduced *S. cerevisiae* iso-1-cytochrome c at 1.23 Å (25) was obtained from the Brookhaven Protein Data Bank (entry no. 1YCC).

All minimization and dynamics studies were carried out on the wild-type structure and the singly modified variants. The consistent valence force field was used for both energy parameters and partial charges on the group charge assignment program. Conjugate gradient was used to evaluate the last 1.5 Å. Minimization proceeded as follows: specific side chains were replaced by methionine with atom positions falling as close to previous positions as possible, methionines were then adjusted by hand to avoid any gross steric clashes. The entire structure was then solvated with a 5-Å layer of explicit water molecules. All atoms in the structure were fixed except for the new methionines, and minimization proceeded by steepest descents for 500 iterations. Side chains were then released and minimized by conjugate gradient for 500 iterations. At this point all atoms were released and minimized by conjugate gradient for 500 iterations, after which these structures were subjected to either minimization by conjugate gradient or to dynamics simulations at 300 K, both for 10,000 iterations. Results were analyzed by comparing the behavior of the wild type and the variants to each other.

**Determination of Physicochemical Properties**

**Spectroscopy**—UV-visible spectra in the range of 750–250 nm were obtained for all mutants using a Beckman DU-65 spectrophotometer. Deviations in the height or position of absorbance bands can give information about how the electronic configuration of mutants might differ from that of the native protein.

**Oxidation-Reduction Potential**—A variation on the “method of mixtures” was used to measure redox potentials of the mutants. This procedure was described in detail by Wallace et al. (26). Various quantities of potassium ferricyanide and ferrocyanide were added to solutions of cytochrome c in 50 mM phosphate buffer at pH 7 in order to make redox buffers of known potential. The state of the redox equilibrium was determined spectrophotometrically by linear regression of the absorbance of the solution at 550 nm and comparison to similar solutions in fully oxidized and reduced states. Ratios of ferrocytochrome to ferricytochrome were plotted against the known ratios of the ferrocyanide to ferricyanide required to achieve these redox equilibria. Extrapolation to the midpoint of the ferricytochrome-ferricytochrome equilibrium gives E’\(_m\) which is equal to the redox potential of the ferrocyanide-ferricyanide couple at the corresponding ratio; this is calculated using the Nernst equation using a value of 0.43 V for E’\(_m\) for the ferrocyanide-ferricyanide couple.

**695-nm Band Titration**—The coordination of ferric iron with Met\(^{80}\) results in a charge transfer band at 695 nm, the intensity of which is used as a measure of heme crevice stability. The sensitivity of the bond

\(^2\) G. D. Brayer, personal communication.

\(^3\) A. R. Fersht, personal communication.
to catastrophic stress makes its loss one of the earliest structural changes with increasing pH (27). Spectrophotometric titrations of this band were carried out by slowly adjusting the pH of solutions of cytochrome c over the range of pH 6–11. The intensity of the 695-nm band was plotted and the pKₐ for the transition calculated. The method used was that described by Wallace (28).

**Fragment Isolation**—Similar techniques were applied to the isolation and purification of the delta mutants to those previously outlined (20, 21, 26). The cleavage reaction was stopped by dilution with water. The mixture was immediately placed on a Sephadex G-50 column (900 × 24 mm) with 7% formic acid as eluant. Fragments were identified on the basis of the elution profile drawn from absorbance at 280 nm. Fragments common to cleavage of all mutants were uncleaved proteins –5 to 103, –5 to 80, and 81 to 103. The remaining three fragments depend on placement of the added methionine: for example, –5 to 25, 26 to 103, and 26 to 80 for the Met² mutant.

In addition, several of the mutants required further purification of desired fragments due to the inability of the G-50 column to separate fragments of similar size completely. Ion-exchange chromatography on a Trisacryl-SP column in a 20–100 mM, pH 7.0 phosphate buffer gradient was required to separate the following fragments: –26 to 103 from –5 to 80, –5 to 35 from 36 to 80, and –5 to 68 from –5 to 80. 7 M urea was added to the phosphate buffer gradient to separate fragments –5 to 80 and 29 to 103 in order to prevent any overlap complex formation that might occur between these two fragments. Immediate desalting on a 1% acetic acid column followed the gradient separation in order to arrest the hydrolysis of the homoserine lactone that occurs under neutral pH conditions and to remove the concentrated urea. Recovered fractions were then freeze-dried.

**Fragment Religation**—Where possible, equimolar amounts of the peptides to be religated were mixed together. These pairs, which correspond to each of the added methionine residues, are as follows: –5 to 25 and 26 to 103; –5 to 28 and 29 to 103; –5 to 35 and 36 to 103; –5 to 55 and 56 to 103; –5 to 68 and 69 to 103; –5 to 75 and 76 to 103. Small amounts of these mixtures were set aside and tested for ascorbate reducibility. Functional complexes, obtained when the break point is N-terminal to residue Trp² (30), and religated cytochromes are ascorbate-reducible, whereas free heme peptides and nonproductive complexes are not (21). The degree of ascorbate reducibility can give information about the tendency of the mixture to reassociate in native-like conformation. The mixtures were then reduced with a few grains of solid sodium dithionite and immediately sealed in airtight glass-walled syringes. After 24-h incubations they were chromatographed on Sephadex G-50. Yields of religated product were calculated from comparing peak areas in elution profiles and from ascorbate reducibility studies of product peaks.

**Characterization of Products**—The products were repurified if yields permitted and freeze-dried. Physicochemical and biological viability of the AFR products was determined by the assays described above and compared to both the native yeast protein and to the protein before AFR experimentation.

**RESULTS AND DISCUSSION**

**Choice of Mutants**

A number of factors influenced the choice of residues for replacement by methionine. The primary motivation was to assay a range of different structural contexts for their influence on the religation reaction. Although cytochrome c contains no genuine β-sheet structures, examples of all other classes of secondary structure, in a variety of tertiary structural settings, are available for exploration. Secondary considerations are that the location be convenient to a putative future semisynthesis, and that mutation of the residue in question should not compromise protein function. To avoid this risk, it would be usual to choose a highly variable residue. The choices we made are shown in Fig. 1superimposed on the α-carbon skeleton of yeast cytochrome c and are described in the following paragraphs.

**Proline 25**—This residue is normally lysine in animal, and glycine in plant species, but varies quite widely in other kingdoms. It lies exposed at the extremity of the first Ω-loop of the protein, residues 18–32, between a β-turn 21–24 and residues 27–29, which is a γ-turn (25).

**Valine 28**—Also surface-exposed, this residue is in the γ-turn. This position can also be occupied by isoleucine, threonine, or glutamine.

**Isoleucine 35**—In contrast, this residue is buried and has conserved hydrophobic character; it will test the inhibitory potential of internal sites in a non-α-helical context, because...
this is the first residue of a β-turn.

**Lysine 55**—Also found as alanine, serine, or arginine, and, indeed, in some higher plants as methionine. The residue is surface-exposed at the neck of the Ω-loop 40–55 between a short stretch of α-helix and a possible 3-residue antiparallel β-structure that involves residues 57–59 (25).

**Leucine 68**—A more risky choice because of its invariance. However, methionine can often substitute for leucine (cf. residues 64 and 98 of cytochrome c), and this residue allows an opportunity to test the suitability of sites at the interface of amphipathic helices. However, because of the presence of the 5-residue N-terminal extension in the yeast protein, this residue is more completely buried than its equivalent in the horse cytochrome. In addition the mutant might shed light on the reason for the invariance of leucine 68.

**Isoleucine 75**—Commonly found as Ile or Val, this residue is Met in *Crithidia* and has been replaced successfully by methionine in another mutant structure (31). It is also the first residue of a β-turn, but although hydrophobic it is only partly buried and provides a comparison with residue 35.

In addition to the specific changes, each mutant, like the original substitution of methionine at position 65 (20) contains replacements of two other side chains. Leucine substitutes for methionine at position 64 to avoid cleavage at this nonproductive site, and Thr replaces Cys at position 102 to obviate dimerization.

### Mutagenesis

All these mutant proteins were extensively tested to establish whether function had been significantly compromised by the introduced change, and in case their properties might reveal new information on the structural or functional role of the individual residues in the cytochrome. In addition, further functional studies were undertaken with the original S65M analog. A preliminary simple, but crude, test is whether the introduced mutant protein will be successfully expressed and support yeast growth on nonfermentable media. All six yeast mutants showed growth rates equivalent to wild-type and yielded $\approx 20$ mg of protein per standard 6-liter culture. However, it should be noted that pNG 4 is a multipolymer vector and minor functional deficiencies in the cytochrome can be overcome by the resulting overexpression of the protein. None of the mutant cytochromes manifested any difference in visible-wavelength electronic absorbance spectra from the parent protein, in either oxidation state, thus indicating an unperturbed coordination sphere.

### Molecular Modeling

Energy minimization of the various mutants showed little change in the structure of cytochrome c in either backbone or side chain positions when compared to the wild type. For the buried side chains 35, 68, and 75, the rearrangement of proximal residues was expected to be more of an issue, whereas the side chains at 25, 28, and 55 are located on the surface and could presumably incorporate the new methionines with a minimum of secondary effects. Since no gross steric conflicts were observed in any of the variants, and because minimization will only find local minima for the structures, the same set of models was subjected to dynamics simulations to see if there might be other aspects of structural alterations that would not otherwise be detected by simple minimization.

During dynamics calculations all of the proteins reached conformational equilibrium by 2 ps. Averaged structures over the time period 2–10 ps were compared to the average structure of the unsubstituted wild type over the same time period, and the RMS deviations of the peptide backbones were evaluated and are shown in Table I. The complete set of aligned structures are shown in Fig. 2. The overlay shows no significant deviations, although there are two noticeable outliers: first, in the N-terminal region and around residues 83–87 of P25M; second, in the region of residues 48–56 for K55M which is in the short helix in the bottom Ω-loop of the protein. An analysis of the hydrogen bonding patterns in all variants showed no significant alterations in hydrogen bonding pattern with the exception of K55M, which loses a hydrogen bond associated with the side-chain amine.

The deviation of the peptide backbone in the P25M variant does not appear to be an effect of the mutation, because it is on the other side of the protein from residue 25 in regions that are assumed to be somewhat disordered normally (25) and this mutant had the smallest deviation observed near the mutation site (see Table I). The low deviation itself is interesting, because it was expected that replacement of the conformationally restricted proline would allow for greater flexibility in this mutant.

The K55M modeling indicates a relatively high structural deviation in the mutation area, so the calculation of deviation was repeated with the other modeling trials to see if this was due to general fluctuations of this region or more specifically to the mutation. Results are shown in column three of Table I and again the K55M variant still has the highest deviation. Fig. 3 shows the α carbon trace for residues 48–79 from both wild-type and K55M simulations with residues 55 and 74 shown explicitly. In all simulations where lysine was not replaced, this residue remained within hydrogen bond distance of the carbonyl oxygen of tyrosine 74. The replacement with methionine at position 55 resulted in a shift in the position of both the tyrosine and the methionine in the K55M simulation, and consequently proximal residues are also shifted. It is possible that substitution of the methionine and the associated loss of this hydrogen bond account for some of the destabilization of the helix and the bottom loop observed during the dynamics simulation, and may also help explain the decreased stability of
this variant to pH denaturation (see below). While methionine is a natural replacement for this residue in rice and other plant cytochromes \( c \), the crystal structure of the rice form also has an additional hydrogen bond from a threonine at position 63 which yeast lacks and which is predicted to add stability (25); this and other sequence differences may compensate for the loss of this specific hydrogen bond in the plant cytochromes \( c \).

### Physicochemical and Biological Characterization of the Mutant Proteins

The packing of the heme group and internal stability of the protein are signaled in a number of ways. Values of midpoint oxidation-reduction potentials, half-times for autoxidation, and the \( pK_a \) of the ligand displacement reaction at high pH are collated in Table II. Redox potential is a crucial functional characteristic of the protein and is subject to modulation by many influences of the protein shell that surrounds the redox center (9). Only in one case, the I75M mutant, does the potential deviate more than \( \pm 12 \) mV from that of the native or C102T form of the cytochrome. The replacement in this case causes a drop of about 30 mV, the same as that seen in the I75M mutant (lacking the M64L change we have made) reported by Rafferty et al. (31). A crystal structure is available for that molecule, which can thus be compared with that of the native protein. Little difference is observed apart from alternative orientations for some of the lysine side chains, which are intrinsically mobile in these structures. The methionine side chain follows the course of the isoleucine it replaces, but, being unbranched, extends further into the interior of the loop that contains it. In the absence of any other obvious cause, we

| Mutant | RMS 1 | Range 2 | RMS 2 | Range 3 | RMS 3 |
|--------|-------|---------|-------|---------|-------|
| P25M   | 0.76  | 15–35   | 0.53  | 45–65   | 0.49  |
| V28M   | 0.81  | 18–38   | 0.83  | 45–65   | 0.84  |
| I35M   | 0.78  | 25–45   | 0.75  | 45–65   | 0.48  |
| K55M   | 0.96  | 45–65   | 0.90  | 45–65   | 0.90  |
| L68M   | 0.84  | 58–78   | 0.72  | 45–65   | 0.82  |
| I75M   | 0.84  | 65–85   | 0.65  | 45–65   | 0.82  |
| M64LL68M | 0.78 | 54–74   | 0.54  | 45–65   | 0.72  |
| M64LL68M | 0.78 | 58–78   | 0.74  |         |       |

### Table II

| Cytochrome | Class of mutated residue | \( E_m^{0} \) (mV) | \( pK_a \) | \( T_{1/2} \) autoxidation (h) |
|------------|--------------------------|---------------------|----------|-----------------------------|
| Native yeast\(^a\) |                     | 279     | 8.5     | 12                           |
| P25M\(^d\) | \( V \)                   | 288     | 8.5     | 22                           |
| V28M       | \( V \)                   | 268     | 8.1     | 15                           |
| I35M       | \( C \)                   | 276     | 8.0     | 20                           |
| K55M       | \( V \)                   | 284     | 7.8\(^e\) | 18                     |
| S65M       | \( V \)                   | 270     | 8.5     | 8                            |
| L68M       | \( I \)                   | 268     | 7.6     | 15                           |
| I75M       | \( C \)                   | 247     | 8.1     | 4                            |

\(^a\) Indicator of internal structural integrity at pH 7.
\(^b\) Indicator of structural resistance of heme crevice to chaotropic conditions.
\(^c\) Includes the C102T mutation to prevent dimerisation.
\(^d\) All mutants also incorporate C102T and M64L changes.
\(^e\) I, invariant; V, variable; C, conservative substitutions only.

\(^f\) Numbers in bold indicate the significant deviations from native values.
propose that the small distortion causes lessens the ability of the loop to shield the bottom of the heme from solvent, and hence lowers redox potential (13, 32).

The pH of loss of the 695-nm Met S–FeIII charge transfer band is a sensitive indicator of the stability of the heme crevice. Part of the function of this structure, in which hydrophobic side chains of the 67–85 loop pack one face of the heme, is to position methionine 80 so that the thioether sulfur coordinates the iron. This is an intrinsically weak interaction for the FeIII state and is susceptible to exchange reactions with both intrinsic and extrinsic ligands. In all mitochondrial cytochromes c there is an isomerization at alkaline pH in which Met80 is displaced by one of two lysine residues (33), concomitant with conformational change and loss of functionality. In two mutants, there is a significant drop in pK for this transition, implying that the replacement does impair crevice stability. In one case, L68M, the side chain is one that packs the heme, and thus the invariance of leucine at this position may be explained by a substantial role in crevice stabilization. In the other, K55M, the molecular modeling results described above may provide an explanation, since the hydrogen bond to the Tyr74 main-chain carbonyl, which is foregone with the substitution of lysine 55, presumably stabilizes the 67–85 loop by linking it to the 39–57 loop, and helps lock the Met80 ligand in place. Although both mutants will occupy largely the normal, functional conformational at physiological pH, the presence of a minority of this inactive form of the protein, in which the heme iron is ligated by lysine, must be considered.

Autodissociation (oxidation of the reduced form by molecular oxygen in solution) is also dependent on the ability of a foreign molecule to penetrate the interior of the cytochrome. The native structure is resistant and half-times of oxidation are lengthy. The bulk of the mutants prepared showed autodissociation times equal to or greater than the native structure (Table II), the one exception being I75M, thus confirming the above conclusion on the effect of this mutation on the structure penetrability by small molecules.

Another important element in cytochrome c function is selectivity of redox partner, mediated by surface conformation and hence charge distribution, since its interactions with physiological partners and membrane surfaces are primarily electrostatic. Changes in dipole moment that would result from conformational change can be detected by changes in chromatographic properties on high performance ion-exchange systems (9). Table III shows that the only significant deviation is exhibited by K55M, where the dipole moment is modified by a change in net charge and not simply distribution. We also checked for effects on the ability to bind to immobilized ATP columns. The ATP binding surface stretches from Arg to Lys72 at the periphery of the “docking ring” of the cytochrome c electron port (34, 35) so that change anywhere in this quadrant may affect retention time on such columns. In practice, the variation seen is small (Table III), mostly implies a stabilization of the interaction between protein and ATP, and is independent of location on the surface. The only change resulting in a significantly shorter retention time occurs with the K55M mutant, and probably reflects the minor ion-exchange chromatographic component in the retention properties of such columns.

Despite the obvious conclusion from the above that the selected changes have near negligible effect on protein structure and stability, some significant deviations in biological properties were recorded (Table IV) that have interesting structure-function implications.

The two assays employed in this study are quite different, although both measure reduction of O2. The succinate oxidase assay employs osmotically shocked and cytochrome c-depleted mitochondria with intact electron transport via complexes II, III, and IV, coupled to phosphorylation. Cytochrome c is added progressively and in these circumstances the limiting transfer is that from reductase (cytochrome c1) to cytochrome c (34). This complex system will not obey standard Michaelis-Menten kinetics, and rates for different cytochromes are compared (Table IV) on the basis of the initial slopes of the O2 consumption curve versus (cytochrome c) curves (36).

The cytochrome oxidase assay uses isolated and purified cytochrome c oxidase, in which this membrane-embedded enzyme complex is detergent-solubilized. Substrate cytochrome c is maintained essentially fully reduced by ascorbate and TMPD, electron transfer rates are measured as O2 consumption curves (36).

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The two assays employed in this study are quite different, although both measure reduction of O2. The succinate oxidase assay employs osmotically shocked and cytochrome c-depleted mitochondria with intact electron transport via complexes II, III, and IV, coupled to phosphorylation. Cytochrome c is added progressively and in these circumstances the limiting transfer is that from reductase (cytochrome c1) to cytochrome c (34). This complex system will not obey standard Michaelis-Menten kinetics, and rates for different cytochromes are compared (Table IV) on the basis of the initial slopes of the O2 consumption rate versus (cytochrome c) curves (36).

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Despite the obvious conclusion from the above that the selected changes have near negligible effect on protein structure and stability, some significant deviations in biological properties were recorded (Table IV) that have interesting structure-function implications.
The drop in relative activity in both assays is even greater for the K55M mutant, yet this lysine residue is distant from the interaction zone. Its substitution will, however, significantly influence the electrostatics of the protein surface, and this property is a major factor in determining both specificity and efficiency of transfer processes (37). It is conceivable that its absence in some species is compensated for by change, either elsewhere in the cytochrome or in the partner. Interestingly, though, chemical modification of Lys\(^{55}\) in the horse protein results in little obvious change in activity (38), so possibly it is the loss of hydrogen bonding potential discussed above, rather than charge difference, that determines the functional change we note.

The effects on activity of methionine substitutions at the other two sites are more readily explicable, on the basis of the change in physical properties. The exchange at the invariant Leu\(^{68}\) that packs the heme face produces the largest shift in \(pK_{a}\) (Table I), so that at pH 7 (succinate oxidase assay) 20% of the mutant protein will be the inactive isomer, and at pH 7.4 (cytochrome oxidase assay), 38%. In addition to this effective depletion, work with semisynthetic mutants of horse cytochrome c\(^4\) shows that the alkaline isomer is actually a potent inhibitor of the oxidase reaction. This structural change can thus fully explain the diminished activities, and indeed the same phenomenon may be in part responsible for the change in the K55M mutant (\(pK_{a}\) = 7.8) discussed above.

At position 75 in cytochrome c conservative changes are acceptable, although as described above, methionine at this position must distort the structure of the bottom loop. In fact this variant has normal activity with oxidase, but is only half as efficient as the native protein in the succinate oxidase system. This property is a probable consequence of the redox potential change that accompanies the structural distortion. Other mutants, in particular those that are distant from the electron port, often exhibit activity losses that are proportionate to \(\Delta E^\circ m\) (13), because this change lessens the thermodynamic driving force (39) to transfer between reductase and cytochrome c, the limiting step in the succinate oxidase assay (28). The relationship established for a set of analogs modified in the bottom loop (13) predicts a relative rate of 55% for a 35-mV drop in potential, consistent with the 48% we determined in this case. While it should be remembered that change in reorganization energy is as influential a factor as change in driving force in determining electron transfer efficiency, this residue is quite far removed from the heme coordination sphere.

Despite the sometimes substantial functional changes noted, none of these mutants is in fact so aberrant as to disqualify it as a starting point for a semisynthetic strategy. But given a preference for a fully functional parent, some rules emerge from this analysis of the set of sites here explored. Although the sample size is one, we have confirmed that invariant residues probably exist for a reason, and that even those for which methionine is a conservative substitution are probably best avoided. In contrast, it appears that evolutionary variability at a site is no great predictor of the complete acceptability of methionine there. It seems that charge change is not well tolerated if it occurs at a surface location; if the site is buried, steric conflicts should be avoided.

\[\text{CNBr Fragmentation}\]

In addition to the inserted residue, cytochrome c has, at position 80, the essential heme-ligating methionine as a potential point of cleavage. Unfortunately, any potential protective effects of ligation are negated by the denaturing low pH conditions of the CNBr reaction. Thus, fragmentation is performed with a suboptimal concentration of CNBr, so that an adequate proportion of the extended fragment containing intact Met\(^{80}\) is obtained. Under these circumstances incomplete cleavage will occur at the other site, and six products are to be expected—uncleaved protein, three limit fragments, and two intermediates. The gel exclusion chromatography elution profiles after CNBr treatment for each of the six new mutants strikingly illustrate the effect of the movement of the introduced cleavage site on the fragmentation pattern, as the heme peptide elutes progressively earlier in the profile with its upward shift in mass from 30 residues in P25M to 80 residues in I75M. One effect of the variability in fragment size is that desirable fragments are not well resolved from undesirable ones in some, though not all, cases. When the peptides were not obtained sufficiently pure for use directly in religation reactions, further separation by ion-exchange methods was employed. The charge/mass ratios of all overlapping pairs were sufficiently different from one another to permit complete resolution (elution profiles not shown).

For **Religation**

The presence of Met\(^{80}\) in the complementary non-heme fragment dictates that the heme fragment is usually obtained in greater quantity than the other peptide, even under limited cleavage conditions. The discrepancy in amounts can be compounded or diminished if prior ion-exchange purification of either component is required. Ultimately this will pose no problem, when the non-heme component is prepared for semisynthesis by solid-phase peptide synthesis (9). In the present trials an excess of heme component was sometimes used: the absolute amounts of each fragment and of functional complex were determined spectrophotometrically using calculated extinction coefficients and the extent of ascorbate reducibility. In general, cleavage reactions were performed on 6-mg (0.5 pmol) samples of each mutant, and hence the amount of noncovalent complex in religation tests was of the order of 1 mg, and reaction volumes were about 0.5 ml. The standard conditions for religation and resolution of coupling mixtures were used and resulted in the yields shown in Table V.

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\[\text{C. J. A. Wallace and I. Clark-Lewis, unpublished results.}\]
These values were calculated from the relative peak areas in the elution profile and the known extinction coefficients at 280 nm of holoprotein product and unligated fragments, using, where there was an inequality, the minority component of the mixture. In some cases (notably I35M and K55M) heme fragment dimers may co-elute on gel filtration with product cytochrome c. Further purification may be infeasible, but since only functional cytochrome can be reduced by ascorbate, where necessary we have used the extent of reducibility to correct the apparent yield downward.

The benchmark yield in this religation reaction is the 60% regularly experienced in the horse 1–65:66–104 system with equimolar quantities of heme and either natural or synthetic non-heme fragments. When translated into the yeast context, religation at the same site, in the S65M mutant, occurred in 50% yield (20). None of the present set of mutants equaled this efficiency, although several approached it. Two sites resulted in relatively poor yields—the buried residues 35 (<10%) and 68 (14%). Religation at the partly or completely exposed residues 25, 28, 55, or 75 was in the 32–40% range. This marked disparity confirms the previous deduction that, if nucleophilic penetration into the protein interior, the reaction will be disfavored.

The relative uniformity of efficiency between surface-exposed sites implies that other factors, the primary or secondary structure contexts in which the breakpoints are found, are much less important. One final criterion in the choice of site is that the product, which now has homoserine at the cleavage/religation position, should have good functionality. Results of succinicate oxidase assays are also reported in Table V as well as the comparable electron-transfer activities of the methionine-containing parents. Generally, there is close accord between the two. The exceptions are the Hse<sup>35</sup> analog, with an activity of only one-third of the Met<sup>35</sup> parent. But this is one of the buried residues and is evolutionarily conservative: naturally, only Ile and Leu, and occasionally Val, Phe, or Ala are found at this position. It is thus likely that Hse would not be well tolerated at this heme-packing location. In contrast, homoserine at residue 75 restores near normal activity. We proposed above that the extra length of the methionine residue distorts the protein coat at the bottom of the heme. This strain would be relieved in the case of homoserine substitution, since this residue lacks the terminal methyl group.

We have secured answers to the questions we earlier posed concerning the autocatalytic religation process and learned a lot about how to direct it in a useful semisynthetic strategy. The constraints on residue choice are now clear, and unless we were extremely astute in our selections it is likely that we have considerable latitude in that choice. Of those we have canvassed, the original position 65 is perhaps the most valuable through a combination of high yield, full activity, and an established routine of synthesis of analogs of the non-heme peptide. However, residue 25 has almost equally good characteristics, and permits access to an additional 40 residues of the sequence not previously easily accessible by semisynthesis, and for analogs modified at residues C-terminal to 75, semisynthesis could readily utilize the more economically synthesized 76–103 peptide. These routes will be exploited to make yeast cytochrome analogs for functional and crystallographic studies.

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