Functional Role of Heme Ligation in Cytochrome c
EFFECTS OF REPLACEMENT OF METHIONINE 80 WITH NATURAL AND NON-NATURAL RESIDUES BY SEMISYNTHESIS*

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The nature of the axial ligation to heme iron has been suggested to be the major determinant of the oxidation-reduction potential of a particular cytochrome, but natural cytochromes that vary significantly in $E_m$ invariably differ from one another in many ways. We proposed to clarify this issue by engineering many different ligation patterns within the same basic molecule, mitochondrial cytochrome c. Since many of the potentially informative substitutions require non-coded amino acids, semisynthesis was the approach we chose, and solid-phase peptide synthesis was used to make a set of nine 39-residue peptides that have been incorporated by autocatalytic fragment religation into the structure of horse cytochrome c. An additional two analogues modified at this position were made by chemical modification of the whole protein. As well as looking at the effect on reduction potential, we examined the effect of varying the ligand sphere on the efficiency of the autocatalytic fragment religation reaction, on the conformation of cytochrome c, on its spectroscopic properties, and in promoting electron transfer between heme c and other redox centers. Substitute residues were chosen to put sulfur, selenium, oxygen, and nitrogen, or even no ligating atom at all in the place of methionine sulfur. We found both subtle and dramatic alterations in spectral properties, which were informative about changes in internal structure and stability brought about by the modifications and which may be useful in identifying novel natural ligation patterns. An unexpected finding was that alanine 80 cytochrome c acquires a hemoglobin-like spectrum, and binds $O_2$ most effectively. Reduction potential changes of $>300$ mV with nitrogen, $>400$ mV with oxygen, and $>300$ mV with thiol sulfur ligation were observed, confirming that variation of the ligand sphere is indeed the most effective way in which the protein coat may modulate the potential of the redox center it encloses. Finally, we obtained more evidence that this axial ligand plays an active role in electron transfer and discovered that histidine could be even more effective in this role.

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This paper is dedicated to Professor R. J. P. Williams, F.R.S. on the occasion of his retirement from the Inorganic Chemistry Laboratory and Wadham College, Oxford University.

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Cytochrome c is a heme-containing electron carrier of the mitochondrial respiratory chain, in which its primary role is to shuttle electrons between the proton-pumping, membrane spanning, multicenter complexes III (where it interacts with cytochrome c) and IV (redox partner cytochrome a). Unlike them, cytochrome c is small, hydrophilic, and mobile, and has proved very amenable to study, so that it has become the favored model for investigation of biological electron transfer. Useful reviews of the physicochemical and biological properties of the protein have been written by Margoliash and Schejter (1986), Dickerson and Timkovich (1975), Salenme (1977), and Pettigrew and Moore (1987, 1988), and recently high resolution crystal structures of both horse and yeast proteins (the mostly widely studied forms) have been published (Louie and Brayer, 1990; Bushnell et al., 1990).

The protein is quite highly positively charged and has a pronounced dipole moment. The positive pole coincides with the point at which the heme edge is exposed on the protein surface, so that this electron port is electrostatically oriented towards the cytoplasmic side of the inner mitochondrial membrane. In fact, it is believed that this interaction is sufficiently strong that cytochrome c remains in loose association and moves between c (reductase) and a (oxidase) by two-dimensional diffusion. When collision occurs, cytochrome c is already productively oriented.

In such a situation it is of necessity a primary role of the protein coat that it provide specificity; short circuits of the respiratory chain are highly undesirable. This specificity is furnished by complementary electrostatic features on cytochrome c and its physiological partners. Another paramount attribute of an efficient member of a chain of electron carriers is an appropriate and stable reduction potential. There is an orderly progression of potential along the chain, with greater or lesser drops within and between carriers, depending on whether work is done. In the mitochondrion, three classes of cytochrome are found, each one populating a distinct region of the sequence, so that electrons flow from b-type (range $-50$ mV) to c-type (200-280 mV) to a-type (280-550 mV) cytochromes. As well as exhibiting slight differences in the chemical nature of the heme group substituents, the three classes also display different patterns of axial ligation.

What determines the reduction potential of a given cytochrome? Given the discrete locations of the three classes, it is natural to propose that a, or the, major factor is the nature of the heme axial ligation (Moore and Williams, 1977). However, when cytochromes outside the mitochondrial chain are considered, the ranges of each class become much wider and overlap to a considerable degree. It is thus clear that other factors may be of great, or even greater, significance, and the relative importance of these possible influences has been widely debated.

The other factors suggested to contribute to the setting of potential are the hydrophobicity of the heme environment.

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(Kassner, 1973), solvent exposure of heme (Stellwagen, 1978),
a specific interaction between heme and Trp^{10} (Myer et al.,
1979), surface charge and internal dielectric constant (Rees,
1980), and special electrostatic interactions (Moore, 1983).

The problem that all these authors faced in attempting to
assess, by comparison of natural cytochromes, the relative
contribution of these factors to establishing potential, was
that those cytochromes that displayed significant quantitative
differences invariably differed from one another in several
qualitative aspects. Therefore, to isolate each of the factors
for such assessment we have embarked on a program of
engineering variations in individual parameters into a single
model, the cytochrome c of horse.

We have already used semisynthesis to examine the effects
of solvent exposure (Wallace and Proudfoot, 1987; Wallace,
1987) and of surface charge (Wallace and Cortesey, 1986,
1987) and special electrostatic interactions (Proudfoot and
Wallace, 1987). The polarity of the heme crevice has been
modified by ourselves and others (Ten Kortenaar et al., 1985;
Wallace et al., 1989). Site-directed mutagenesis of the yeast
protein has also been useful in examining some of these
factors (Cutler et al., 1989; Louie et al., 1988). Subtle and, less
often, gross changes have been introduced, and changes rang-
ing from a few to over one hundred millivolts have resulted,
thus suggesting that these factors are more likely to have been
used by evolution in fine-tuning the potential than in deter-
mining the range in which a given cytochrome will operate.

The goal of the present study is therefore to obtain a
definitive view of the role of the ligand sphere in setting the
reduction potential of a heme center. The low spin iron of
cytochrome c is octahedrally coordinated, the most common
arrangement for transition metals in the 2+ and 3+ oxidation
states, with four ligands provided by the planar pyrrole nitro-
gens and the axial ligation furnished by histidine imidazole
nitrogen (residue 18) and methionine thioether sulfur (residue
80). Of these the most accessible by established semisynthetic
routes is Met^{15} (Wallace et al., 1989). The natural set of amino
carboxylates contains some other potential ligands: Lys N, Arg N,
Cys S, and possibly Ser/Thr O, Glu/Asp O, and Tyr O. A far
greater diversity is available in non-coded form, making semis-
synthesis the ideal tool for the replacement of Met^{15} by
interesting alternative ligands.

The technology of solid-phase peptide synthesis (SSPS)\(^1\)
is sufficiently well developed that fragments as large as 39
residues can be prepared with confidence (Wallace et al.,
1989), allowing one to use autocatalytic fragment religation
(AFR) with naturally obtained (1-65)H. This approach to
protein reassembly (Proudfoot et al., 1989; Wallace, 1991)
relies on the proximity and orientation imposed on the termini
at the breakpoint in a complex of large fragments to effect
catalysis of peptide bond formation. It is vastly simpler and
more efficient than the more familiar chemical approaches to
peptide bond synthesis. We have again combined SSPS and
AFR to introduce a range of potential ligating, and some non-
ligating, groups in place of the side chain of Met^{15}, with the
experimental goals set out below. The substitutes chosen were
cysteine, alanine, histidine, selenomethionine, thienylalanine,
S-methylcysteine, ornithine, cyanoalanine, norleucine, ho-
moserine, and methionine sulfoxide (Fig. 1). The latter two
analogues were prepared by chemical modification of the native
protein.

Since we refined this approach to the engineering of cyto-
crome c structure in the 66-104 region, others have used it,
and because residue 80 is an obvious target for structure-
function studies, have also made changes at this position. The
first to report replacement of methionine were Raphael and
Gray, who inserted histidine (Raphael and Gray, 1989). Since
then we have made a preliminary report of the preparation of
some of the above analogues (Wallace and Clark-Lewis, 1991),
concurrent with a further report (Raphael and Gray, 1991) of
the semisynthesis of [Cys^{15} and [Leu^{15}]proteins.

The present paper describes a detailed study of the prop-
erties of 11 position 80 analogues, which had a number of
goals. We wished first to examine any effect of the residue at
position 80 on the efficiency of AFR, since it has been sug-
gested that the greater affinity of Fe^{2+} as compared with Fe^{3+}
for thioether sulfur is responsible for the low efficiency of
recombination in complexes kept in the oxidized state (Har-

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\(^1\) The abbreviations used in the text are: SSPS, solid-phase peptide
synthesis; AFR, autocatalytic fragment religation; HPLC, high per-
formance liquid chromatography; TFA, trifluoroacetic acid; SeMet,
selenomethionine; S-MeCys, S-methylcysteine; Thr, thienylalanine;
CNAla, cyanoalanine; Nle, norleucine; Hse, homoserine; MeSO,
methionine sulfoxide.

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FIG. 1. The structures of the amino acid side chains intro-
duced at position 80 by semisynthesis or chemical modifica-
tion. The \(\alpha\)-carbon atoms of all residues are aligned to show the
spatial relationship of side chain and iron atom (top) if there were no
change in backbone conformation.
burly, 1978). We also wanted to look at the influence of novel axial ligand combinations on spectroscopic properties of heme proteins at physiological pH, and how sensitive those properties are to environmental factors. The data will permit an estimation of the ligand field strength of these groups within proteins and may aid the classification of newly isolated heme proteins. The prime objective was, of course, to quantitate the proteins and may aid the classification of newly isolated heme proteins. The yield of a semisynthetic cytochrome analogue obtained in the reassociation process was judged by comparing the height of the holocytochrome peak with those of fragments 1–65 and 66–104 in the elution profile of gel exclusion chromatography. These yields are compiled in Table I and fall in a range from very low to as high as that obtained with the native sequence. This result contrasts with previous experience (Wallace et al., 1989) and implies that residue 80 has some role, direct or indirect, in assisting the reassociation process. Such a conclusion was reached by Harbury upon consideration of the difference between reassociation efficiencies in oxidized and reduced states (Harbury, 1978), although he proposed that it was the relative strength of the ferrous iron-thioether sulfur bond that provided the major driving force to productive reassociation.

Indeed, we find that in all but one of the cases studied (Table I), the oxidized form of the complex, no matter what the replacement residue at position 80, does not efficiently promote reassociation. This implies that some other aspect of the subtle conformational differences that distinguish the oxidation states, rather than the strength of the coordinate bond, is responsible for the differential productivity. However, if cysteine is the substitute residue at position 80, a high level of recombination occurs even when the complex is left in the oxidized state, so that it is conceivable that the process is assisted by (in this case) the presumed strong affinity between ferric iron and the thiolate anion. However, the picture is confused by the observation that different products result from reassociation in different oxidation states (see below).

Previous studies (Corradin and Harbury, 1971; Harbury, 1978) have shown that recombination is a consequence of the adoption of the native protein conformation by the noncovalent complex and that the structural requirements are very stringent. The need for a very close fit of the two fragments has been experimentally confirmed (Wilgus et al., 1978; Wallace et al., 1986). Any structural change at residue 80 that leads to distortion of the cytochrome fold, unless compensated by other thermodynamic factors, would shift the equilibrium away from productive complexation.

Thus both the thioether homologues of methionine, S-methylcysteine and thienylalanine, would place the normal conformation under strain, by diminishing the distance between the peptide backbone and the sulfur atom, or by increasing bulk in the heme crevice, respectively. A similar argument could be advanced to explain the low productivity of the CNAla''-containing complex (Fig. 1).

However, as a strict homologue of methionine, norleucine should not be distorting. Our observation of the low spin character of the complex and product (see below) suggests that some other ligand is provided by the protein and that norleucine may not occupy the normal Met' position in the structure of the complex, resulting in low reassociation yields, whereas the much more compact alanine could accommodate the ligand replacement without itself being displaced.

Finally, the histidine analogue is only produced in low yield. Although the side chain is more bulky than methionine, others have reported the efficient semisynthesis of this analogue (Raphael and Gray, 1989). We have repeated the synthesis of the fragment without improving reassociation yields. On both occasions we obtained a homogeneous product that satisfied all analytical criteria, and did give a cytochrome with physicochemical properties that matched those reported by Raphael and Gray (1989). We have been unable to discover the reasons for this discrepancy in apparent yields, despite varying buffer composition and pH.

A consequence of the low yields is that relatively small amounts of purified products were available for further study in the S-MeCys''', CNAla''', Thi''', His''', and Nle'' cases, and hence experimental attention has focused on the high yielding analogues.

**Analytical and Preparative Chromatography of Semisynthetic Products**

Because of the rapid autoxidation of the semisynthetic analogues, or the low yields in which they were obtained, most products underwent a single ion-exchange purification step. The effectiveness of the preparative column in removing contaminants, primarily polymeric forms of the fragment (1–65)H, polymers of the cytochrome itself, cytochrome in which the heme is degraded, or deaminated forms of the cytochrome, were checked by analytical chromatography and by the UV-visible spectroscopic properties of the final products. Fig. 3 shows examples of reversed-phase HPLC elution profiles, clearly indicating the homogeneous nature of the products and the lack of deaminated material that characterizes even the best commercially available natural cytochromes. The retention times compiled in Table III show shifts that are consistent with the nature of the induced substitution, since the protein is presumed to lack a stable tertiary structure in the acetonitrile/trifluorosacetic acid gradient used for elution. Cation-exchange HPLC does not cause denaturation so that elution position reflects both overall charge on the protein and its distribution. Surface conformational change can cause change in the dipole moment and, hence, retention time. Thus these data can give information not just on the purity of the products, but also on their state. Fig. 4 shows some examples; the profiles generally show doubled peaks, due to reduction by an unknown mechanism in the strictly anaerobic chromatographic system. The data (not shown) reveal that the majority of the analogues elute at positions identical to those of the parent protein. Those exhibiting significant differences are [Cys'''] and [Ala''']cytochromes. Some conformational change is implied, although the reduced form of [Ala''']cytochrome c elutes at the standard position.

Interestingly, those analogues that involve protonatable
Residue 80 Mutants and Heme Ligation in Cytochrome c

TABLE V

Features of the UV-visible spectra of cytochrome c and residue 80 analogues at pH 7

<table>
<thead>
<tr>
<th>Cytochrome</th>
<th>Ferric state</th>
<th>Autotidizable</th>
<th>Ferrous state</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Charge transfer</td>
<td>(\alpha/\beta)</td>
<td>Soret</td>
<td>(\delta)</td>
</tr>
<tr>
<td>Native, state III (pH 2.5–9.5)</td>
<td>Yes</td>
<td>529</td>
<td>408</td>
<td>360</td>
</tr>
<tr>
<td>State II (pH &lt;2.5)</td>
<td>No</td>
<td>523</td>
<td>394</td>
<td>350</td>
</tr>
<tr>
<td>State IV (pH &gt;9.5)</td>
<td>No</td>
<td>530</td>
<td>405</td>
<td>354</td>
</tr>
<tr>
<td>Orn(^{a})</td>
<td>No</td>
<td>529</td>
<td>403</td>
<td>354</td>
</tr>
<tr>
<td>Ala(^{a})</td>
<td>No</td>
<td>535</td>
<td>403</td>
<td>350</td>
</tr>
<tr>
<td>Ala(^{a},O_{2})</td>
<td>691 nm</td>
<td>532</td>
<td>412</td>
<td>363</td>
</tr>
<tr>
<td>Nle(^{a})</td>
<td>No</td>
<td>529</td>
<td>406</td>
<td>354</td>
</tr>
<tr>
<td>CNAla(^{a})</td>
<td>No</td>
<td>528</td>
<td>406</td>
<td>350</td>
</tr>
<tr>
<td>Thienyl Ala(^{a})</td>
<td>No</td>
<td>530</td>
<td>405</td>
<td>353</td>
</tr>
<tr>
<td>His(^{a})</td>
<td>660?</td>
<td>528</td>
<td>406</td>
<td>350</td>
</tr>
<tr>
<td>Cys(^{a}) isomer I</td>
<td>530</td>
<td>404</td>
<td>352</td>
<td>Yes</td>
</tr>
<tr>
<td>Cys(^{a}) isomer II</td>
<td>640–740</td>
<td>539</td>
<td>417</td>
<td>356</td>
</tr>
<tr>
<td>Hse(^{a})</td>
<td>No</td>
<td>535</td>
<td>403</td>
<td>353</td>
</tr>
<tr>
<td>Hse(^{a},O_{2})</td>
<td>570</td>
<td>536</td>
<td>403</td>
<td>349</td>
</tr>
<tr>
<td>S-MetCys(^{a})</td>
<td>683</td>
<td>527</td>
<td>403</td>
<td>357</td>
</tr>
<tr>
<td>MetSO(^{a})</td>
<td>No</td>
<td>528</td>
<td>404</td>
<td>353</td>
</tr>
</tbody>
</table>

substitute residues, [Orn\(^{a}\)] and [His\(^{a}\)]cytochrome c, also have elution times close to those of the native protein. Thus either the base is not protonated, essential for coordination of heme iron, or the coincidental results reflect simultaneous charge and conformation changes. The spectroscopic data discussed below imply that these two residues do indeed provide a heme axial ligand.

Surface conformation can also be monitored by ATP affinity chromatography. Cytochrome c has, at intermediate ionic strength, a single ATP binding site of moderate affinity and high specificity (Craig and Wallace, 1991). This site incorporates arginine 91 and thus lies at the (conventional) upper left side of the molecule, close to residue 80. Since, by its nature, it probably involves several side chains, conformational change in that region is likely to reduce affinity and shorten transit time on the immobilized ATP column. Table IV shows results for a number of analogues; of these, it is the [Cys\(^{a}\)] and [Ala\(^{a}\)]mutants that show a loss of ATP binding affinity, while [SeMet\(^{a}\)] and [Orn\(^{a}\)]proteins are unaffected.

Functional Properties of the Analogues

The UV-visible spectroscopic data obtained for the engineered cytochromes are compared with those for native horse cytochrome c in Table V, and the midpoint oxidation-reduction potentials are collated in Table VI. Bioassay results are presented in Table VII. Discussion of these, and other results, follows; they will be considered in groups based on the atom provided for ligation.

Selenomethionine 80

The replacement of sulfur by selenium represents a minimal change, but nonetheless differences in properties ensue. Spin state is unaffected, but there are small shifts (3–4 nm) in the major absorbance maxima in the visible spectrum (Table V) toward the red, although the near-IR ferric iron-ligand charge transfer band is slightly increased in energy from 695 to 691 nm. This band disappears with rising pH, as some conformational change causes the bond to break, with a pK of 9.25 for horse cytochrome c in 50 mM phosphate buffer. In the analogue, the pK of this transition is 9.2, implying that the change has not destabilized the heme crevice structure. The reduction potential is decreased by nearly 50 mV. Since the atomic radius of covalently bonded selenium differs little from sulfur (1.16 Å versus 1.02 Å), and spectroscopic and chromatographic techniques detect no differences in conformation we may assume that this marked drop is not due to a major disruption of the heme crevice, but to a difference in electronic properties of the coordinating atom.

In the absence of any change in spin-state, Moore and Williams (1977) enumerated three primary electronic factors influencing reduction potential. In terms of these parameters, however, selenium differs little from sulfur (for example its Pauling electronegativity is 2.4 versus 2.5 for sulfur), although in the characteristic H-bond between it an$ and the TyP OH might be changed from the normal 3.2 Å. This link connects the length of the iron-ligand bond may be changed by the above noted atomic radius difference. It is however possible that it is the other interactions in which sulfur (or selenium) participates that modulate the ligand properties. For example, the characteristic H-bond between it and the Tyr\(^{77}\) OH might also be changed from the normal 3.2 Å. This link connects the methionine sulfur to an internal H-bond relay that is suggested to have a role in determining reduction potential (Takano et al., 1981b; Bushnell et al., 1990). A clear understanding of the processes responsible for the change will depend on a detailed structural determination. In the succinate oxidase assay system, selenomethionine 80 cytochrome c had a specific activity 44% that of native. This is much in
provided by thioether sulfur. This is indeed the case, although we have discovered that with cysteine two products with quite different properties are obtained, depending on the conditions under which fragment relaxation is performed. The product from the normal, highly reducing conditions, which we term isomer I, has most similarity to the parent protein in terms of spectroscopic properties in the neutral to alkaline range. As the pH drops there is a transition to a high spin form with pK 6.63 in 50 mM phosphate buffer.

Isomer II is formed when fragments are recombined under oxidizing conditions. Radically different spectroscopic properties result and the spectrum is unaffected by pH change in the range 3–11. It is this form that matches the Cysso analogue reported by Raphael and Gray (1991) and which they likened to a cytochrome P450. This material has a reduction potential no greater than −140 mV and is only very slowly reduced by dithionite, suggesting a very low value (Raphael and Gray (1991) report −390 mV). If the reduced form is reoxidized in air, the result is a spectrum that no longer resembles that of isomer II, but looks like isomer I. This form of the analogue has a much higher potential of about −65 mV. This interconvertibility of the two forms, and their identity on reversed-phase HPLC in denaturing conditions, implies that the difference lies in the tertiary, rather than the covalent structure. This view is supported by the differences noted above in nondenaturing analytical chromatographic properties. The radical spectroscopic differences imply very different coordination spheres. Raphael and Gray (1991) have proposed Cys-Fe-His coordination for isomer II, on the grounds that Cys-Fe(III) ligation can be observed by magnetic circular dichroism spectroscopy and the spectrum and the reduction potential resemble the thiolate ligated cytochrome P450, and the assumption that the normal Fe-His ligation is not disrupted. One difficulty with this line of reasoning is that the heme iron of P450 is coordinated by Cys/H2O, not Cys/His, and one might reasonably expect some significant differences with this latter ligation pattern. If, as we believe, the ligation pattern in isomer II is Cys-Fe-OH2, then some conformational rearrangement involving the displacement of histidine 18 must be involved. Certainly, our chromatographic studies of surface conformation show Cysso isomer II to deviate most from the native structure.

The reduced forms of isomer I and II have identical spectral properties, and both autoxidize to ferric isomer I, suggesting that they are indeed identical. If so, then reduction of isomer II must require some conformational change, explaining the very low reduction potential and the very slow reduction by dithionite.

Under extremes of pressure, cytochrome P450 acquires a cytochrome c-like spectrum2 that is thought to be due to novel heme coordination by the distal histidine brought about by compaction of the structure. If true, this observation suggests that it is isomer I that has the Cys-Fe-His ligation pattern, and that the transition to a high spin form with pK 6.6 is a consequence of protonation of thiolate to the more weakly coordinating thiol. Alternatively, like the [Ala80]cytochrome c analogue discussed below, the sixth ligand of isomer I might be provided by some other residue, or a water molecule in an unusual environment. The similarities in the pK values of the high spin transition (Table V) might support this view; however, there is a marked difference in λmax for the α band between [Cysso]isomer I and [Ala80]cytochrome c analogues.

Neither isomer binds CO in the oxidized state, but the reduced protein does so at neutral pH, indicative of weak ligation by cysteine of ferrous iron. The absorbance spectrum

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2 J. Kornblatt, personal communication.
of the carbon monoxide complex of [CySCyt]cytochrome c does not resemble that of CO-cytochrome P450, implying that it is cysteine, not histidine, that is displaced by the extrinsic ligand.

**Nitrogenous Ligands: Ornithine 80, Histidine 80, and Cyaanoalanine 80**

Although both ornithine and histidine would normally be extensively protonated at pH 7, there is substantial evidence that they are not and are buried in the hydrophobic heme crevice, ligating the iron via nitrogen atoms. The chromatographic data described above suggests no charge change in the analogues, and both materials have distinctive low spin spectra, a property they share with the third nitrogenous analogue, [CNAAla]cytochrome c (Table V).

Naturally, all three lack the 695 nm methionine sulfurrer ferric iron charge transfer band, although [His60]cytochrome c shows a novel band centered at 660 nm. The near-IR spectrum of [Orn60]cytochrome c is featureless, so this absorbance may be specific to interaction with imidazole nitrogen.

The view that in alkaline conditions the iron of ferricytochrome c is coordinated by amino nitrogen is commonly held (Gadsby et al., 1987) despite strong evidence against it (Wallace and Corthesy, 1987). We have looked for clues in the spectrum of [Orn60]ferricytochrome c as to which position is correct. Although the Met → Orn substitution does produce changes in the absorbance maxima that mirror those seen on the alkaline transition for α/β, Soret, and δ bands, most of the substitutions we have introduced, nitrogenous or not, produce similar shifts. One way in which [Orn60]cytochrome c differs substantially from state IV, the alkaline form, of native cytochrome c is in the stability of coordination. The prevailing ligation in the latter is easily displaced by cyanide; we find that even exposure to 100 mM KCN for 5 h results in only partial formation of the cyanide complex of [Orn60]ferricytochrome c. This is not yet a conclusive argument against amino nitrogen ligation in state IV, since a significant conformation change also occurs in the alkaline transition.

Another feature of the [Orn60]ferricytochrome spectrum is an increase in the extinction coefficients of the Soret (from 1.1 × 105 M⁻¹ cm⁻¹ for the parent protein to 1.4 × 105 for [Orn60]cytochrome c) and α/β bands. Although the height of the Soret band is increased in state IV of the native protein, that of the α/β band decreases. Similarly, in the reduced state, Soret and α bands substantially increased in absorbance, although the β band remains unaffected.

In [CNAAla]cytochrome c, some distortion of the heme crevice structure would be necessary to accommodate the coordination geometry; the nitrogen lone pair, the CN triple bond, and the C−CN bond have to be approximately in line. This may explain the low yield of this analogue relative to the [Orn60]cytochrome c product, but does not apparently prevent coordination, for an entirely typical low spin spectrum in both oxidation states results.

The reduction potential of [CNAAla]cytochrome c is about −100 mV, and that of [Orn60]cytochrome c about −40 mV. We determined a value of 30 mV for [His60]cytochrome c, which compares favorably with the 41 mV reported by Raphael and Gray (1989). This spread within a set of analogues in which the coordinating atom is always nitrogen must be explicable in terms of the varied nature of the bonding that the nitrogen atom undertakes. Nonetheless it is clear that in general nitrogen as a ligand gives a much lower potential than uncharged sulfur. Both [CNAAla] and [Orn60]cytochrome c derivatives have very low activity in the succinate oxidase assay system, in line with expectations based on their reduction potentials (Wallace and Proudfoot, 1987). [His60]cytochrome c would also be expected to have limited electron transfer capability. In fact our observation is of relatively high efficiency at limiting cytochrome c concentrations (Fig. 5). Furthermore, we noted that this specific activity increased with increasing time of storage in solution at 4 °C (Table VII), paralleling the observation of a time-dependent increase in E° (Raphael and Gray, 1989). The finding that [His60]cytochrome c is so active is startling. The change could influence the relative affinities of the two oxidation states for the redox partners, but in the absence of any evidence for a change in conformation or dipole moment this seems less likely than a direct effect on the displacement of the electron between redox centers. This observation reinforces our suspicion that the sixth ligand residue might play a role in helping surmount the activation barrier to transfer.

**Oxygen-containing Ligands**

Both homoserine 80 and methionine sulfoxide 80 analogues can be obtained by chemical modification of the native protein in substantial yield. [MetSO0]cytochrome c has been studied in detail by Myer's group (Pande et al., 1987; Myer et al., 1987). We confirmed the isolation of two isomers of the analogue by ion exchange chromatography, with retention times on cation-exchange HPLC of 10.0 and 10.6 for the ferrous forms of the analogues versus 11.3 for ferricytochrome c, and 10.7 and 11.1 for the ferric forms versus 12.2 for native ferricytochrome.

They concluded that in the neutral to alkaline pH range, the low spin spectrum is a consequence of coordination of heme iron by the sulfoxide oxygen, and that the two conformers of methionine sulfoxide result in two isomers of the protein containing it. This coordination is lost with pK values for the transition to a high spin form of 6.1 and 6.6 (Myer et al., 1987).

Like them, we observed that [MetSO0]analogue are reducible by ascorbate, and reoxidized in air, although both processes are slow. Upon oxygenation, the 695 nm band is not restored, nor is there evidence of other spectroscopic anomalies. We find reduction potentials of 205 and 203 mV, quite different from those previously determined (175 and 135 mV), but this difference is probably attributable to two factors. Our determinations were made in the standard 50 mM phosphate buffer, pH 7, by a spectroscopic titration with ferricyanide. Pande et al. (1987) used a potentiometric method.
in 20 mM cacodylate buffer containing a mixture of mediators. This latter approach gave a value for the native protein of 242 mV, some 18 mV less than the generally accepted value for \( E'_{\text{m}} \) for cytochrome c of 260 mV (Table VI). This difference alone can reconcile the anomaly for one isomer, and it may be that our buffer composition has a stabilizing effect on the lower potential analogue.

With reduction potentials of this order, a substantial electron transfer capacity could be expected. In fact the specific activity of both isomers in the succinate oxidase system is low, confirming the observations of Pande et al. (1987) that the analogues performed poorly in assays of reductase activity.

The properties of the [Hse]
\(^{35}S\)cytochrome are very different. Although the oxidized form is substantially low spin at neutral pH, its \( \alpha/\beta \) band is red shifted to 535 nm, and there is a transition to a high spin state with pH 6.1. The reduced form has considerable high spin character at neutral pH, with a weak \( \beta \) band and a 390/415 split in the Soret region. The inference must be that if indeed the hydroxyl oxygen of homoserine coordinates the iron, it is easily displaced by water. When the dithionite-reduced analogue is buffer-exchanged by gel exclusion into an oxygen-containing medium, novel bands at 570 and 536 nm are revealed, characteristic of O₂ binding to ferrous heme. These bands decay within a few hours to restore the characteristic ferric heme spectrum. [Hse]
\(^{35}S\)Cytochrome c has \( E'_{\text{m}} \) of less than \(-100\) mV, and in consequence negligible biological activity. Thus the difference in coordination properties of hydroxyl and sulfoxide oxygen can lead to at least a 300-mV change in reduction potential. The considerations of Moore and Williams (1977) predict that the lower partial negative charge on the sulfoxide oxygen and its unsaturated character should combine to give a higher potential.

**Aliphatic Hydrocarbons**

Being neither good donors nor acceptors, saturated carbon atoms do not coordinate metal ions. Nevertheless, both norleucine 80 and alanine 80 cytochromes c show low spin spectral properties in the ferric state at neutral pH, although the [Ala]
\(^{35}S\)protein goes high spin with pH 6.55.

Clearly the protein itself provides an alternative strong field ligand, as in the alkaline transition to state IV in the native protein. Whether it is the same ligand remains an open question, for although there are spectral similarities between [Nle]
\(^{35}S\)cytochrome c and state IV, [Ala]
\(^{35}S\)cytochrome c is distinctive. [Nle]
\(^{35}S\)cytochrome c retains low spin character in the ferrous form, but the \( \beta \) band of ferrous [Ala]cytochrome c collapses, and the Soret band doubles, with a novel absorbance at 430 nm. This hemoglobin-like property is confirmed by oxygenating the reduced protein, which acquires bands at 571 and 536 nm (Fig. 6). Unlike the [Hse]
\(^{35}S\)protein, oxy-[Ala]
\(^{35}S\)cytochrome c is very stable. Autodissociation is not significant on standing overnight, nor does overnight exposure to water aspirator pressure or chromatography in fully aerobic conditions completely deoxygenate the analogue. In creating a hole in the protein structure adjacent to the iron atom, by truncating the side chain of residue 80, we have induced in the cytochrome an oxygen binding site of considerable affinity and transmuted it to a hemoglobin. The considerable stability of the oxocytochrome is reflected in the reduction potentials of the analogue. \( E'_{\text{m}} \) for the Fe\(^{3+}/\text{Fe}^{2+} \) couple is 185 mV (comparable with 150 mV for Hb or 50 mV for Mb), but 220 mV for the Fe\(^{3+}/\text{Fe}^{2+} \)O₂ couple, so the oxy form is stabilized by 0.8 kcal mol\(^{-1} \) relative to the unoxigenated protein.

[Nle]
\(^{35}S\)cytochrome c does not bind O₂ and has a reduction potential little different from that of the native protein. Being isosteric with methionine should allow its introduction with minimum disruption to the heme crevice, although the evidence of alternative coordination suggests that it cannot exactly occupy the normal methionine conformation. The [Leu]
\(^{35}S\)analogue (Raphael and Gray, 1991) also appears to have a low spin spectrum, at least in the ferric form, but a much diminished \( E'_{\text{m}} \) of \(-45\) mV. Possibly the branched side chain is more disruptive of heme crevice structure.

Although the [Nle]
\(^{35}S\)protein has substantial biological activity, neither it nor the [Ala]
\(^{35}S\)protein show electron transfer rates in concordance with their relatively elevated reduction potentials. This disparity may be related to the spin-state change in [Ala]
\(^{35}S\)cytochrome c, but the behavior of the [Nle]
\(^{35}S\)cytochrome c analogue is further evidence of the facilitating role of the ligand in electron movement.

**Conclusions**

We find that the efficiency of the autocatalytic reigation of the complex of fragments (1-65)H and (66-104) of horse cytochrome c is dependent on the nature of the residue at position 80, although no clear pattern of causation has emerged. The reaction is strongly promoted by the adoption of the reduced conformation, although this is unrelated to the presence of a Met S-Fe²⁺ bond. Adequate material was obtained for biological and spectroscopic studies of all analogues. In five cases (SdMet, Cys isomer 1, Cys isomer 2, Ala, and Orn) yields are sufficient for more detailed studies of products, including future crystallographic structure determination.

We conclude that the nature of the axial ligation to the iron atom probably is the most quantitatively significant factor in determining the oxidation-reduction potential of protein-enclosed heme. Replacement of Met by other thioether or selenoether ligands leads to at most limited change, but nitrogenous ligands at this position result in 250-350-mV potential drops. Hydroxyl oxygen produces an even greater change, and thiolate can shift the midpoint potential by more than 600 mV.

Unexpectedly, ligand replacement also results in more or less pronounced changes in absorption spectra, particularly
in the visible region. The spectrum of a cytochrome (Orn⁶⁰⁰) in which the axial ligand is amino nitrogen differs in significant ways from that of the alkaline form of the native protein, even though lysine amino nitrogen has been suggested as the ligand in this state.

The spectral data also reveal that some analogues with nonligating or weakly ligating residues become at least partially pentacoordinate and high-spin in the reduced state. This coordination pattern resembles hemoglobins and myoglobin, and, like them, these analogues when exposed to O₂ develop an oxymyoglobin like spectrum. In [Ala⁶⁰⁰]cytochrome c, where truncation of the side chain should leave a "hole," oxygen is tightly bound; in the [Hse⁶⁰⁰]cytochrome c analogue, with a significantly larger residue, binding is less tight.

The electron transfer efficiencies of some analogues show deviations from the values that would be expected if the ligand change influenced driving force (the reduction potential difference between centers) alone. Where surface conformation change is observed, as in [Ala⁶⁰⁰] or [MetSO⁶⁰⁰]cytochrome c, a change in affinity for redox partners is possible; where the conformation is unchanging, the ligand change must have affected the activation barrier to transfer. In the case of [Nle⁶⁰⁰]cytochrome c, like previous examples of deviation, the "conductivity" of the transfer pathway is reduced. Unusually, when histidine replaces methionine, an increase in conductivity results. This result may have important implications for our understanding of biological electron transfer, and further investigation of the properties of the [His⁶⁰⁰]cytochrome c analogue will be undertaken.

Acknowledgments—We are grateful to Dr. Sandy Kieland of the University of Victoria, British Columbia, for performing the amino acid analyses, and Angela Brigley-Cole, Phillip Owen, and Gregory Radigan for expert technical assistance.

REFERENCES
Kassner, R. J. (1973) J. Am. Chem. Soc. 95, 2674–2677

Continued on next page.
Experimental Procedures

Materials

N-terminal. b-hydroxybenzooyl (BzH) amino acids were obtained from the Peptide Institute, Osaka, Japan except N-benzyloxycarbonyl (Boc) amino acids and N-benzylloxycarbonyl (Boc) cysteine. Boc-cysteine and Boc-cysteine hemithioacetal were obtained from peptide Research, Pittsburgh, PA. N-benzylloxycarbonyl-L-cysteine and N-benzylloxycarbonyl-(N-hydroxybenzyl) cysteine which were obtained from Skymed Inc. 2.9 mg of purified cytochrome c from calf heart was purchased from Calbiochem., San Diego. Fraction 5 (Tris buffer, pH 7.4) of 10 mg protein/mL was purchased from FPLC (Pharmacia, Piscataway, NJ) and fraction 7 (buffer, pH 6.0) of 10 mg protein/mL was purchased from BioRad Laboratories, Inc. (Richmond, CA). Read pressure was set to 0.5 MPa and flow rate was set to 0.7 mL per minute. Methanol was used as the gradient solvent from 0% to 100% over 10 minutes.

Preparation of Protein

Cytochrome c, 2.5 mg, was reconstituted using a solution of sodium phosphate (50 mM, pH 7.4) and 2 M urea. The solution was incubated at 37°C for 24 hours. The reconstituted protein solution was filtered through a 0.22 μm filter. The filtrate was then loaded onto a SP-Sepharose cation-exchange column (2.5 mL volume) and washed with 100 mM sodium phosphate buffer, pH 7.4, containing 1 M NaCl. The protein was eluted with 500 mM sodium phosphate buffer, pH 7.4, containing 1 M NaCl. The eluate was concentrated and dialyzed against Tris buffer, pH 7.4.

Protein Synthesis Methods

Cytochrome c, 25 ΜM, was reconstituted using a solution of sodium phosphate (50 mM, pH 7.4) and 2 M urea. The solution was incubated at 37°C for 24 hours. The reconstituted protein solution was filtered through a 0.22 μm filter. The filtrate was then loaded onto a SP-Sepharose cation-exchange column (2.5 mL volume) and washed with 100 mM sodium phosphate buffer, pH 7.4, containing 1 M NaCl. The protein was eluted with 500 mM sodium phosphate buffer, pH 7.4, containing 1 M NaCl. The eluate was concentrated and dialyzed against Tris buffer, pH 7.4.

Selenium Oxidation Reactions

The residues in some of the mutants were challenged by oxidation to potential selenites. In most cases aliquots of concentrated solutions of a selenite (e.g., KCN) were added to solutions of proteins in 2.5 M urea in Tris buffer, pH 7.4. For reactions with CO, the reactions were first reduced with sodium dithionite, and then buffer-exchanged on a short column of Sepharose Cl-4B, equilibrated with a buffer containing 0.1 M NaCl and 0.1 M Tris buffer, pH 7.4. The eluate was monitored for the appearance of peroxides formed by reoxidation of CO.

Spectral Characteristics of the Analogues

The absorbance of the intact 750-nm band was recorded on a Beckman model DB-6 spectrophotometer. Any deviation from the characteristic absorbance maxima and extinction coefficients of the analogues can give information on both the structural and electronic state of the cytochrome. pH-titration was performed using 10 mM Tris buffer, pH 7.4, and other samples were also titrated at pH 5.0 and 7.0. The absorbance was monitored at 450 nm and 600 nm. The absorbance at 405 nm was also monitored during pH titrations. The absorbance at 578 nm was used as a marker for the presence of selenocysteine.

Oxidation-Reduction Potentials of the Analogues

Reduction potentials of pK were determined by the method of Wallace et al. (1985). We used an apparatus which fits to the standard of the cytochrome c-redox couple (initial redox potential, 7.450 for the cytochrome-containing buffer was a solution of 50 mM Tris buffer, pH 7.4, containing 0.1 M NaCl and 0.1 M Tris buffer, pH 7.4). The method was originally described by Cockcroft et al. (1970). This product was then used to separate the cytochrome c-redox couple by electrophoresis using a polyacrylamide gel, and the relative mobility for ATP synthase was measured as a function of the relative mobility for ATP synthase.

Biological Assays

The efficiency of the analogues in restoring C. subtilis to cytochrome c-depleted E. coli was determined by complementation of three different methylotreres (Wallace and Proudfoot, 1978).

Results and Discussion

Peptide synthesis

The chain assembly for the 39 residue cytochrome c analogues (66-104) was obtained from an average yield of 30% per step as monitored by the coupling reaction using uracil (Sann et al., 1980). The efficiency of the analogues was optimized by the coupling reaction using uracil (Sann et al., 1980). The efficiency of the analogues was optimized by the coupling reaction using uracil (Sann et al., 1980). The efficiency of the analogues was optimized by the coupling reaction using uracil (Sann et al., 1980). The efficiency of the analogues was optimized by the coupling reaction using uracil (Sann et al., 1980).

Identification of the Analogues

The analogues were identified by mass spectrometry using a high-performance liquid chromatography (HPLC) system. The analogues were identified by mass spectrometry using a high-performance liquid chromatography (HPLC) system. The analogues were identified by mass spectrometry using a high-performance liquid chromatography (HPLC) system. The analogues were identified by mass spectrometry using a high-performance liquid chromatography (HPLC) system.

Table I: Amino Acid Composition of the Synthetic Cytochrome c 66-104 Analogues

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>3</td>
</tr>
<tr>
<td>Glu</td>
<td>2</td>
</tr>
<tr>
<td>Ser</td>
<td>2</td>
</tr>
<tr>
<td>Thr</td>
<td>1</td>
</tr>
<tr>
<td>Gly</td>
<td>1</td>
</tr>
<tr>
<td>Val</td>
<td>1</td>
</tr>
<tr>
<td>Leu</td>
<td>1</td>
</tr>
<tr>
<td>Met</td>
<td>1</td>
</tr>
<tr>
<td>Pro</td>
<td>1</td>
</tr>
<tr>
<td>Cys</td>
<td>1</td>
</tr>
<tr>
<td>Hyp</td>
<td>1</td>
</tr>
<tr>
<td>Hyp</td>
<td>1</td>
</tr>
</tbody>
</table>

Table II: The Inhibition of the Reductase Position 60 of Fragment 66-104 on the Efficiency of Autocatalytic Fragment Conduction in Cytochrome c

<table>
<thead>
<tr>
<th>Fragment 66-104 Analogue</th>
<th>Maximal Religation Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeL+ Complex</td>
<td>FeL+ Complex</td>
</tr>
</tbody>
</table>

Native (Met) 66-104  | 60%                     | 60%                     |
S-Met(Cys)66-104      | 40%                     | 40%                     |
[Fe(CN)]6-66-104      | 40%                     | 40%                     |
[Selenocysteine]66-104| 40%                     | 40%                     |
Hyp66-104              | 40%                     | 40%                     |
Phe66-104              | 40%                     | 40%                     |
Val66-104              | 40%                     | 40%                     |
Leu66-104              | 40%                     | 40%                     |
Met66-104              | 40%                     | 40%                     |
Cys66-104              | 40%                     | 40%                     |
Hyp66-104              | 40%                     | 40%                     |
Hyp66-104              | 40%                     | 40%                     |
TABLE III
A comparison of retention times of residue 80 analogues of horse cytochrome c on reverse-phase HPLC. Waters 600E system fitted with a Deltapak C8 column, elution by a gradient of acetonitrile-water (0:100 - 60:40) containing 0.1% TFA.

<table>
<thead>
<tr>
<th>Cytochrome</th>
<th>Retention time (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column I:</td>
<td></td>
</tr>
<tr>
<td>Horse Cytochrome</td>
<td>40.4</td>
</tr>
<tr>
<td>SeMet80</td>
<td>39.9</td>
</tr>
<tr>
<td>Cys80 Isomer I</td>
<td>38.8</td>
</tr>
<tr>
<td>Cys80 Isomer II</td>
<td>37.6</td>
</tr>
<tr>
<td>Ala80</td>
<td>42.6</td>
</tr>
<tr>
<td>Column II:</td>
<td></td>
</tr>
<tr>
<td>Horse cytochrome</td>
<td>50.8</td>
</tr>
<tr>
<td>S-Met Cys80</td>
<td>58.0</td>
</tr>
</tbody>
</table>

TABLE IV
Comparison of retention times of cytochrome c and analogues on an immobilised ATP affinity gel (Pharmacia AGATP). Data from two separate columns are shown. Cytochrome samples are loaded onto the gel in 10mM phosphate buffer, pH 7.00, and eluted with 35mM phosphate buffer, pH 6.95.

<table>
<thead>
<tr>
<th>Cytochrome</th>
<th>Retention time (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column I:</td>
<td></td>
</tr>
<tr>
<td>Horse cytochrome</td>
<td>24</td>
</tr>
<tr>
<td>SeMet80</td>
<td>28</td>
</tr>
<tr>
<td>Cys80 Isomer II</td>
<td>7</td>
</tr>
<tr>
<td>Column II:</td>
<td></td>
</tr>
<tr>
<td>Horse cytochrome</td>
<td>34</td>
</tr>
<tr>
<td>Orn80</td>
<td>39</td>
</tr>
<tr>
<td>Ala80</td>
<td>22</td>
</tr>
<tr>
<td>Cys80 Isomer I</td>
<td>11</td>
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</tbody>
</table>