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Substitutions Engineered by Chemical Synthesis at Three Conserved Sites in Mitochondrial Cytochrome *c*

THERMODYNAMIC AND FUNCTIONAL CONSEQUENCES*

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Analogues of the 39-residue CNBr fragment of horse cytochrome *c* (66–104) have been prepared by total chemical synthesis. Conformationally assisted ligation of these peptides with the native cytochrome *c* fragment 1–65 (homoserine lactone form) occurred in high yield. Semisynthetic protein molecules of the expected molecular weight were obtained that had folded structures similar to the native molecule as shown by spectral properties and by cross-reactivity with a panel of monoclonal antibodies sensitive to the three-dimensional integrity of cytochrome *c*. Point mutations were introduced into the horse sequence at three strongly conserved sites: Tyr^{67}, Thr^{78}, and Ala^{83}. The contributions of these 3 residues to the stability of the heme crevice were estimated by titration of the 695 nm absorption due to coordination of ferric iron by the sixth ligand methionine sulfur. The roles of these residues in catalysis of electron transfer and in establishing the value of the redox potential of cytochrome *c* were also investigated. The hydroxyl group of Tyr^{67} modulates the spectral properties of the heme and has a profound influence on its redox properties, but hydrogen bonding involving this phenolic hydroxyl does not stabilize the heme crevice. In contrast, we find that Thr^{78} is strongly stabilizing and that asparagine is not an adequate substitute for this residue because of the greater entropic cost of burying its side chain. The low biological activity of analogues modified at this position, despite normal redox potentials, imply a role for Thr^{78} in the electron transfer mechanism. The replacement of Ala^{83} by proline induces a similar phenomenon. An involvement of this residue in the catalysis of electron transfer provides an explanation of the low reactivity of plant mitochondrial cytochromes *c* in mammalian redox systems.

The functional roles undertaken by proteins in biological systems are very numerous. The capacity for this diversity lies in the variety of chemical groups available in the natural set of amino acids and the potential for varied folding patterns that can bring together novel combinations of these functional groups. To fully understand and potentially manipulate those biological activities we require a comprehensive view of the principles of structure-function relations in proteins, which may only be obtained by rational engineering of well understood protein structures. For this purpose and to increase our understanding of redox proteins, cytochrome *c* should prove a paradigm, as it has for studies of molecular evolution (Dickerson and Timkovitch, 1975), immunogenicity (Cooper *et al.*, 1987; Paterson, 1989), and electron transfer (Salemme, 1977), because of the wealth of structural and functional information already available and because the protein encapsulates a wide range of characteristic functions.

Specific protein analogues for structure-function studies can be engineered either by direct modification of the structure of the protein itself or of the gene that encodes it. The two approaches are complementary, and the method of choice will be dictated by the experimental objectives. In the case of the cytochrome *c* molecule, a wide range of "mutant" proteins are available from natural sources (Dickerson and Timkovitch, 1975). Structure-function correlations can be drawn from comparison of the properties of these variant molecules with the amino acid sequence differences between them, although that information is limited by the evolutionary pressure to conserve optimal function. Both natural and site-directed mutants of the yeast protein are now also being used for mechanistic studies of cytochrome *c* (Hampsey *et al.*, 1986; Pielak *et al.*, 1985, 1986; Liang *et al.*, 1987).

An alternative approach to the generation of structural variants in proteins is semisynthesis (Offord, 1987). This technique is of particular value if the experimental objectives include the insertion of a site-specific reporter group (Busch *et al.*, 1985) or of a noncoded amino acid (Wallace and Corthésy, 1986), or a radical reorganization of protein structure (Wallace, 1987). Semisynthesis consists in essence of the limited fragmentation of a natural protein, the modification of the structure of the fragment that contains the residue(s) of interest, and the condensation of the modified peptide and the unmodified fragments to give the correct sequence of the target protein.

Cytochrome *c* is undoubtedly the protein that has received most attention from practitioners of semisynthesis (Barstow *et al.*, 1977; Koul *et al.*, 1979; Ten Kortenaar *et al.*, 1985;

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Wallace *et al.*, 1986), because of the propensity to spontaneous resynthesis exhibited by the noncovalent complex of CNBr fragments 1–65 (homoserine lactone form) and 66–104 (vertebrate species numerotation) (Corradin and Harbury, 1974). The adoption by the complex of a near-native conformation (Corradin and Harbury, 1971) catalyzes the aminolysis of the C-terminal homoserine lactone at residue 65 by the α-amino group of residue 66. The conformational requirements for this type of reaction are stringent (Wallace *et al.*, 1986; Proudfoot *et al.*, 1989), so a functional purification is achieved by a selection of those molecules competent to assume the native conformation.

Among the techniques that have been employed for fragment modification prior to religation in the cytochrome *c* 1–65 plus 66–104 system are sequential degradation and resynthesis (Wallace and Corthésy, 1986), treatment of fragments with side chain-specific reagents (Wallace and Rose, 1983), or use of a natural fragment from a homologous protein (Wallace *et al.*, 1986); but conceptually the simplest is replacement of the 66–104 fragment by a totally synthetic peptide. Due to methodology limitations, this approach had been of only limited utility in the past. There has been only a single report of the use of a fully synthetic fragment 66–104 prepared by solid phase methods (Barstow *et al.*, 1977), and most workers have employed the condensation of smaller fragments prepared by solution synthesis to create 66–104 (Koul *et al.*, 1979; Ten Kortenaar *et al.*, 1985; Wallace *et al.*, 1986). Resynthesis yields of synthetic analogues have generally been low relative to those achieved with naturally obtained peptide fragments, presumably because of low purity of the synthetic fragments. Recent developments in the field of solid phase peptide synthesis (Kent, 1988) have made possible the routine preparation of large synthetic fragments in good yield and purity. This led us to suppose that the yield of semisynthetic analogues of cytochrome *c* could be improved using unequivocally synthesized 66–104 fragments.

We have used this chemical peptide synthesis-protein semisynthesis approach to prepare analogues of horse cytochrome *c* incorporating modifications at three strongly conserved residues: Tyr^{67}, Thr^{78}, and Ala^{83}. Previous studies have imputed a functional role for each one (Takano and Dickerson, 1981b; Wallace and Boulter, 1988). In the case of Tyr^{67} and Thr^{78} we have substituted the naturally occurring amino acid side chain with one that bears the same carbon skeleton but lacks the hydrogen-bonding hydroxyl group. For example, phenylalanine replaces tyrosine at 67, and α-aminobutyric acid replaces threonine in position 78. Thr^{78} was also replaced by asparagine, as found uniquely in *Chlamydomonas* cytochrome *c* (Amati *et al.*, 1988). Ala^{83} was replaced by proline, a substitution specific to, and universal in, plant cytochromes *c*. The question of additivity of the effects of individual point mutations was examined by the preparation of a cytochrome *c* double mutant, incorporating substitutions at both position 78 and 83 in the active site Ω-loop (Wallace, 1987). As a control for our synthetic procedures, we also synthesized the fragment 66–104 of a natural animal mitochondrial sequence and used this in the preparation of semisynthetic [Hse^{65}] cytochrome *c*.[1]

Here we report the preparation and characterization of these analogues of the cytochrome *c* molecule, and the investigation of the effects of these changes on the physicochemical, biochemical, and biological properties of the mutant proteins.

EXPERIMENTAL PROCEDURES AND RESULTS AND DISCUSSION[2]

Design of Experiments—The nature of the association between the two fragments leads to an acceleration of the reaction between the α-amino group of fragment 66–104 and the lactone (internal ester) of C-terminal homoserine of 1–65, that in many ways models enzyme catalysis (Proudfoot *et al.*, 1989). The high yield from equimolar mixtures at low concentration, under mild conditions, makes the condensation process economical in materials and free of side reactions. A further advantage of this conformationally directed religation over traditional methods of fragment condensation in protein synthesis is the lack of a requirement for side chain protection and subsequent deprotection. Thus for substitutions in the 66–104 sector of the sequence the use of the spontaneous resynthesis method is the method of choice. The product proteins will differ from authentic natural cytochrome *c*, apart from the desired replacements, in that they will contain a homoserine residue at position 65, in place of methionine. Position 65 is a highly variable site in mitochondrial cytochromes *c*, and a number of studies have shown that [Hse^{65}]cytochrome *c* is functionally indistinguishable from the parent protein (Corradin and Harbury, 1974; Boswell *et al.*, 1981; Wallace and Rose, 1983).

In the past, analogues of the segment 66–104 have usually been prepared by chemical modification of the 39-residue fragment prepared by cleavage of native cytochrome *c* (*e.g.* Wallace and Corthésy, 1986). Portions of the 66–104 sequence have been prepared chemically and ligated to fragments prepared from native cytochrome *c* (Koul *et al.*, 1979), but total chemical synthesis has been used only rarely (Barstow *et al.*, 1977). Until recently, the chemical synthesis of a 39-residue peptide in good yield and high purity was a major undertaking. However, recent advances in stepwise solid phase synthesis have made the unequivocal synthesis of peptides of this length a more reasonable task. Simultaneous progress has occurred in the technology of peptide purification by preparative reverse phase HPLC, and in the covalent characterization of large synthetic peptides, especially by mass spectrometry. This increased accessibility of large peptides was used to advantage in the total chemical synthesis of cytochrome *c* (66–104) according to the native horse sequence, and of a series of analogues incorporating specific amino acid substitutions (Fig. 1).

The analogues were designed to address the following questions. The crystallographic studies of Dickerson's group (Takano and Dickerson, 1981a, 1981b) have implicated both Tyr^{67} and Thr^{78} in a hydrogen bond network within the crevice at the conventional left face of the porphyrin ring (Fig. 2). The role of this network is unclear; it may be involved in closing the crevice or in stabilizing the weak interaction of the sixth ligand methionine sulfur with the ferric iron, or even involved in the catalysis of electron transfer, as suggested by Takano and Dickerson (1981b). The substitutions of phenylalanine at position 67 and Aba at position 78 are intended to investigate

[1] The abbreviations used are: Hse, homoserine; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DMF, *N,N*-dimethylformamide; HOBt, 1-hydroxybenzotriazole; DIEA, *N,N*-diisopropylethylamine; Boc, *t*-butyloxycarbonyl; Z, benzyloxycarbonyl; Bzl, benzyl; Tos, toluenesulfonyl; TFA, trifluoroacetic acid; Aba, L-α-aminobutyric acid; HPLC, high pressure liquid chromatography. All other amino acid abbreviations are in accord with standard IUPAC nomenclature.

[2] Portions of this paper (including "Experimental Procedures," part of "Results and Discussion," Figs. 3–5, 7, 8, 10, and 11, and Tables I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

Native	Glu^{66}-**Tyr**-Leu-Glu-Asn^{70}-Pro-Lys-Lys-Tyr-Ile-Pro-Gly-**Thr**-Lys-Met^{80}-		
Phe^{67}	**Phe**	-	
Asn^{78}	-	**Asn**	
Pro^{83}	-	-	
Asn^{78}, Pro^{83}	-	**Asn**	
Aba^{78}	-	**Aba**	

-Ile^{81}-Phe-**Ala**-Gly-Ile-Lys^{86}-Lys-Lys-Thr-Glu^{90}-Arg-Glu-Asp-Leu-Ile-Ala-Tyr-Leu-Lys-Lys^{100}-

-
-
Pro
Pro
-

-Ala^{101}-Thr-Asn-Glu

FIG. 1. **The amino acid sequence of the 66–104 fragment of horse cytochrome *c*, showing in *bold* the sites of substitutions and the replacements made.**

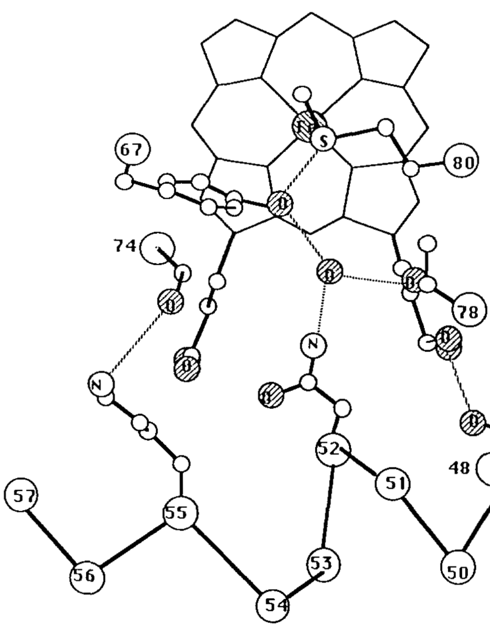

FIG. 2. **The network of hydrogen bonds formed by amino acids at the methionine sulfur-ligated face of the heme in both ferri- and ferrocytochrome *c*.** Included in this structure are the hydroxyl groups of both tyrosine 67 and threonine 78 and a buried water molecule that links them (Takano and Dickerson, 1981b).

the consequences of the elimination of the hydrogen bonds these side chains are involved in. Thr^{78} was thought to be invariant, but the recent sequence analysis of the *Chlamydomonas* cytochrome *c* gene has revealed that in this species alone, asparagine is present (Amati *et al.*, 1988). Since this residue should also be competent to participate in hydrogen bonding we set out to ascertain why this substitution did not occur more frequently, by introducing it into the horse protein and examining the effects on the properties on the mutant cytochrome *c* molecule produced. Residue 83 is part of the hydrophobic stretch that lines the exposed heme edge in the center of the active site. This sequence includes phenylalanine 82, proposed to participate as an intermediary in electron transfer between heme centers (Wendeloski *et al.*, 1987). Residue 83 is alanine or valine in all animal species, glycine in yeasts and Protista, and proline in all plants. This variation has been proposed to account for the functional difference between plant and animal mitochondrial cytochromes *c* (Wallace and Boulter, 1988). We wished to test this proposal by the introduction of proline into an animal cytochrome.

Peptide Synthesis—The target sequences shown in Fig. 1 were prepared in side chain-protected form by stepwise solid phase peptide synthesis (Merrifield, 1963). See Miniprint for details.

Religation and Characterization of Semisynthetic Proteins—Gel filtration on Sephadex G-50 of the products of coupling fragments 1–65 and synthetic 66–104 gave peaks eluting at the volume expected of cytochrome *c*. Peak heights on the elution profile were measured as a means of calculating coupling yields, which are set out in Table I. The analytical data obtained with samples of the synthetic fragments employed in semisynthesis had shown them to be pure and compositionally correct: the high quality of the peptides is further attested to by the uniformly high yields obtained in the conformation-dependent coupling, comparable in all but one case to that seen with native fragments. The exception, when using [Aba^{78}]66–104, may be due to a lower stability of the complex with fragment 1–65, since this will depend on the same forces that stabilize the whole protein. Of the analogues prepared, only [Aba^{78}] is significantly less stable at pH 7 and room temperature than the native protein.

Cation-exchange chromatography was employed for the purification of the cytochrome *c* peak from gel filtration. In the oxidized state, crude products were resolved into one major and, generally, a number of minor components (Fig. 4*a*). A further minor component was eliminated by ion exchange of the reduced protein (Fig. 4*b*). In every case the major component eluted at the same point as the corresponding native cytochrome *c*. None of the substitutions involve a charge change. Most heme-containing fragments of the protein, and even denatured protein with the normal covalent structure, elute at different ionic strengths.

Confirmation that the desired covalent structure was formed was also obtained through ^{252}Cf plasma desorption time-of-flight mass spectrometry. The molecular masses of the analogue peaks are included in Table I. These values represent the maxima of broad mass envelopes (Fig. 5) due to natural isotopic heterogeneity as well as in-flight unimolecular decay, so that the experimental uncertainty is estimated to be ±0.2% or ±25 mass units. Thus these data can give an assurance of the purity of the products and that covalent assembly of the complete sequence of cytochrome *c* has been achieved.

The semisynthetic proteins were also characterized by UV-visible spectral properties. The maxima of the principal absorption bands, and the ratios of certain absorbances, at pH 7 are compared in Table II. These ratios are good criteria of purity. For example, the ratio of A_{280}/A_{360} will be high if non-heme proteins or peptides are present. Close identity was observed between semisynthetic and native cytochromes *c*. The only significant differences from the value of A_{280}/A_{360} for the native protein were in the case of the Phe^{67} protein, where the ratio is low as a consequence of the elimination of the contribution of Tyr^{67} to absorbance at 280 nm, and in [Aba^{78}]cytochrome *c*, where the 360 nm band is shifted and intensified.

Contamination by heme-containing peptides is revealed by low ratios of the absorbance of reduced and oxidized forms at 550 nm: in the Phe^{67} analogue the lower absorption of the reduced form at 550 nm is a consequence of the shift of the α-band from 550 to 553 nm. Contaminating heme-containing peptides or proteins are not reduced by ascorbate, but are by sodium dithionite, so that a comparison of the absorbances of the α-band in the presence of the two reductants is a further index of purity. By this criterion all ion-exchanged products were 100% pure.

The identity or close similarity of visible spectra of the semisynthetic proteins to that of native cytochrome *c*, particularly with respect to the very perturbation-sensitive 695 nm band, indicate that in every case the religated polypeptide

chain has assumed the native conformation. This inference is supported by the normal redox potentials of two of the analogues as well as the semisynthetic protein of native sequence. Denaturation is inevitably attended by a drop in redox potential, though this is not the only structural factor that can influence this parameter.

We have also tested four of the semisynthetic analogues with a panel of monoclonal antibodies directed against regions of the surface of cytochrome *c*. These have been shown to be sensitive indicators of conformational integrity, capable of detecting both localized changes in stereochemistry as a consequence of side chain substitution, and long range perturbations of conformation due to changes elsewhere in the structure (Collawn *et al.*, 1988). Fig. 6 sets out the results of testing four analogues with this panel of antibodies. It is clear from these data that no detectable modification occurs within the epitopes of these antibodies, and that, therefore, no significant change to the overall structure of the protein has been induced. The failure of E8 to detect the side chain substitution at Tyr^{67}, which on the basis of the previous mapping studies (Paterson, 1989) falls within its epitope, is no doubt due to the buried nature of this side chain.

Physicochemical Properties of the Semisynthetic Proteins—The UV-visible spectra of the analogues were examined for any deviation, either in wavelength of maximum absorption or height of the principal bands (Table II). Cytochromes *c* of almost all species are characterized by strong bands in the yellow and green regions of the spectrum (ferrocytochrome *c*: α-band, 550 nm; β-band, 520 nm; ferricytochrome *c*: α-band, 528 nm), as well as the Soret band in the blue region common to all heme proteins (ferrocytochrome *c*, 416 nm; ferricytochrome *c*, 410 nm). The 3-nm red shift in the α- and β-bands of [Phe^{67}]cytochrome *c* is the first example of such a spectral perturbation evoked by a change in the polypeptide chain enclosing the heme. Normally, such shifts are a consequence of a change in the structure of the heme prosthetic group itself (Dickerson and Timkovitch, 1975). A possible explanation of this red shift is as follows. The tyrosine to phenylalanine substitution that we have made at residue 67 not only abolishes hydrogen-bonding capability, but also changes the polarity of the side chain. The crystallographic data (Takano and Dickerson, 1981b) show that the hydroxyl group is in close contact with the heme. The red shift in the principal absorption bands seen on substitution of phenylalanine is indicative of a modified heme electronic structure, which we therefore propose to be due to a shift in the electron distribution in the π-cloud of the porphyrin ring (Shelnutt *et al.*, 1981), induced most probably by the localized and proximate change in polarity.

One of the most informative absorption bands in the characteristic spectrum is a weak charge-transfer band centered at 695 nm due to the interaction of the ferric heme iron and the sixth coordinating ligand, the thioether sulfur of methionine 80 (Dickerson and Timkovitch, 1975). It is a most sensitive indicator of perturbation of the protein structure due to rising pH, temperature, or denaturant concentration. Whether the loss of the band that accompanies the changing conditions is a consequence of a ligand replacement reaction,

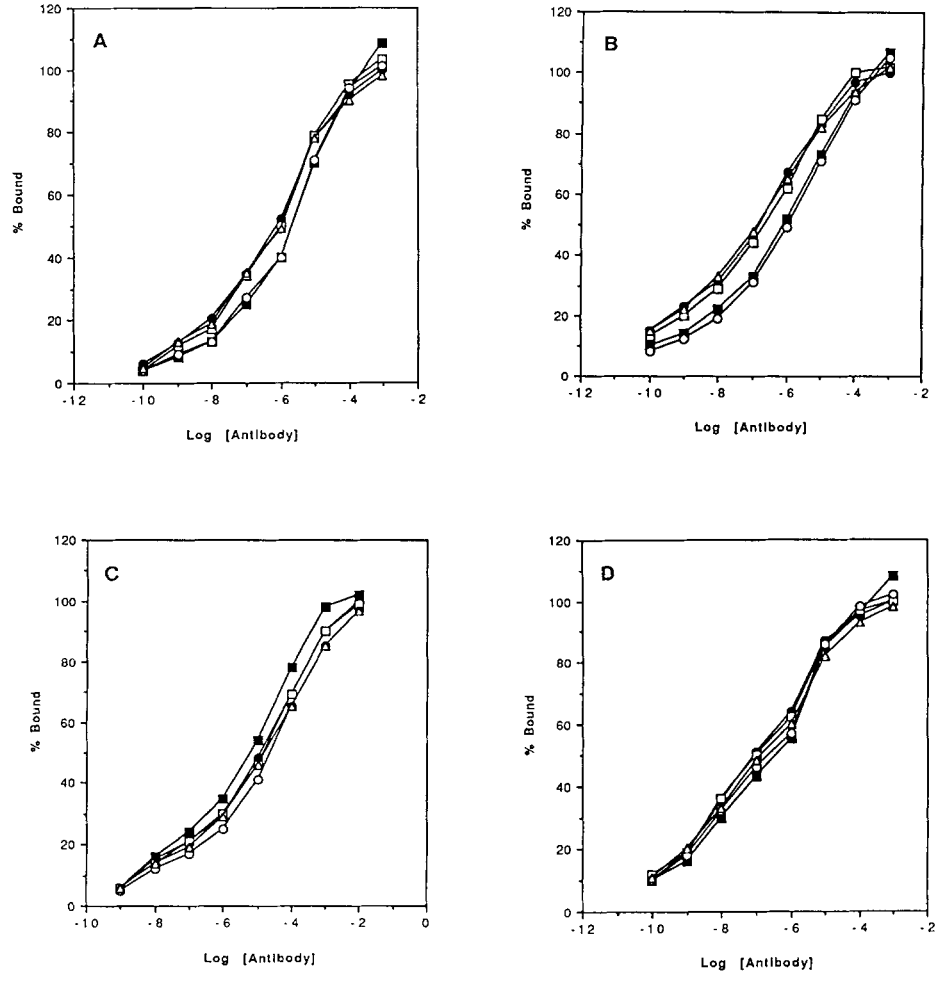

FIG. 6. **Solid phase radioimmunoassay for monoclonal antibody C3 (*A*), C7 (*B*), E3 (*C*), and E8 (*D*) on the cytochrome *c* analogues.** ●, cytochrome *c* (native); ■, Asn^{78}, Pro^{83}; □, Pro^{83}; ○, Aba^{78}; △, Phe^{67}. Nonspecific background radioactivity was subtracted from each of the binding curves.

or of a reorientation of the bond, is still the subject of controversy (Dickerson and Timkovitch, 1975; Pettigrew *et al.*, 1976; Bosshard, 1981; Wallace, 1984; Gadsby *et al.*, 1987; Wallace and Corthésy, 1987).

Changes in heme crevice stability were investigated by pH and thermal titrations. The change in 695 nm absorbance with increasing pH for all analogues is plotted in Fig. 7. Significant variations, both up and down, are seen in the pK values derived from these plots (Table III). Fig. 8 shows curves plotted for the thermal titrations of the band. The direction, order and extent of variation of these from that of the native protein mirror the results of the pH titration. Although it has been suggested that the changes accompanying denaturation differ between the two types of perturbation (MacDonald and Phillips, 1973), our results suggest that the forces that oppose denaturation do not.

The Arrhenius plots derived from the thermal data are shown in Fig. 9, and the thermodynamic parameters calculated from the thermal and pH titrations are compiled in Table III. For the more stable analogues, there is a departure from linearity in the Arrhenius plots at high temperatures immediately prior to precipitation onset. The biphasic form was noted for native cytochrome *c* by Kaminsky *et al.* (1973), who ascribed it to an acceleration of the loss of the iron-sulfur band consequent on histidine ionization and the breaking of the iron-imidazole nitrogen fifth co-ordination bond. Osheroff *et al.* (1980) noted that they did not observe biphasicity but we find that its onset occurs at temperatures higher than employed in their titrations.

We report a transition temperature and thermodynamic parameters for the horse protein at variance with the results obtained by Osheroff *et al.* (1980) (55 °C, $\Delta G'_0 = 1.5$ *versus* 49 °C, $\Delta G'_0 = 1.2$). Since titrations cannot be taken to completion, it is possible that such differences are due to different assumptions of the value of the end point. We have assumed a value equal to that at the alkaline extreme of the pH titration, *i.e.* 0.1–0.2 of the initial value. The approach of Kaminsky *et al.* (1973), used by Osheroff *et al.* (1980), would result in a different value for $\Delta G'_0$, for recalculation of our data using a value of 0.3 for the end point results in differences in the estimate of $\Delta G'_0$ of up to 0.15 kcal mol^{-1}.

However, as is clear from the data in Table III, protein concentration can also have a marked effect on the thermodynamic parameters: because of the weakness of the 695 nm band, previous studies have tended to employ high (>1000 μM) protein concentrations. We give data obtained at three different cytochrome *c* concentrations to demonstrate the concentration effect and to permit effective comparison with the results from our analogue samples. The comparisons shown in Table III reveal a substantial variation in stability, compounded of changes in both entropic and enthalpic contribution of the interactions in which the modified groups participate.

The parameters derived from these measurements can be interpreted in terms of the amino acid substitutions at defined positions in the sequence to produce informative data on the role of specific residues and interactions that affect the stability of tertiary structure. Residue 67 is tyrosine in all known mitochondrial cytochromes with the single exception of that of *Euglena*. The involvement of this residue in an internal hydrogen bonding network (Fig. 2) suggested a role in structural stabilization (Takano and Dickerson, 1981b). It is clear from our data that Tyr^{67} makes no net contribution to protein stability. The phenylalanine-containing analogue shows greater resistance to denaturation than the native protein (Figs. 7 and 8; Table III); the contribution to $\Delta G'_0$ of $\Delta H'_0$ of hydrogen bond formation in the native protein is outweighed at 37 °C by the entropic cost of burying the polar hydroxyl group (Table III).

In contrast to native cytochrome *c*, and indeed to all the other analogues presently described, the Phe^{67} protein is autoxidized relatively rapidly at neutral pH. The oxidation by molecular oxygen of ferrocytochrome *c* is thermodynamically favorable, and it has been proposed that the native protein structure provides a kinetic barrier to this process that is overcome in general denaturation of the protein (Margoliash and Schejter, 1966) or in functional but destabilized two-fragment complexes of the protein (Wallace and Proudfoot, 1987). However, since the present data show that the Phe^{67} substitution does not destabilize the heme crevice, but that the analogue is rapidly oxidized, it may be that the hydroxyl group of Tyr^{67} plays a specific role in blocking access of molecular oxygen to the ferrous iron atom in the native protein.

The analogues modified at residue 78 show, under standard conditions (pH 7, 25 °C), weakness in the 695 nm absorbance band (Figs. 7 and 8). This result confirms a report (Ten Kortenaar *et al.*, 1985) that the Val^{78} analogue lacked this band. In fact, the pH and thermal titrations show that any weakness at pH 7 and 25 °C is a consequence of shifts in pK and T_m and that at lower temperature and pH the band can be restored to its characteristic height. The weakening of the

TABLE III

Thermodynamic parameters for loss of the ferric heme iron-methionine sulfur coordination bond

$\Delta G'_0$ for the alkaline transition is calculated from the pK as described by Osheroff *et al.* (1980). $\Delta H'_0$ and $\Delta G'_0$ for the thermal transition are obtained from the Arrhenius plots in Fig. 9, as described, and $\Delta S'_0$ by using these two values in the expression $\Delta G'_0 = \Delta H'_0 - T\Delta S'_0$.

Cytochrome *c*	Thermal[a]				pH[b]	
	$T_{1/2}$	$\Delta H'_0$	$\Delta S'_0$	$\Delta G'_0$	pK	$\Delta G'_0$
	°C					
Horse cytochrome *c* (1000 μM)	55[c]	16.1	49	1.47		
Horse cytochrome *c* (170 μM)	64	20.6	61	2.36	9.25	3.0
Horse cytochrome *c* (60 μM)	65	21.1	62	2.45		
[Phe^{67}]Cytochrome *c* (250 μM)	107	9.8	25	2.07	10.65	5.0
[Pro^{83}]Cytochrome *c* (50 μM)	53	21.1	65	1.77	8.95	2.7
[Asn^{78},Pro^{83}]Cytochrome *c* (50 μM)	48	19.9	62	1.38	8.25	1.7
[Asn^{78}]Cytochrome *c* (25 μM)	35	21.0	68	0.64	8.10	1.5
[Aba^{78}]Cytochrome *c* (50 μM)	12	14.7	52	−0.65	<7	<0

[a] In 10 mM sodium cacodylate, 10 mM sodium chloride, pH 7.
[b] In 40 mM potassium phosphate.
[c] Osheroff *et al.* (1980) report 49 °C for an unspecified concentration.

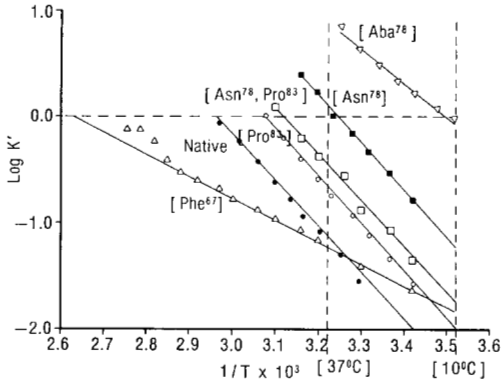

FIG. 9. **Arrhenius plots of the equilibria between "cold" (695 nm band present) and "hot" (lack of 695 nm band) forms of cytochrome *c* and analogues.** The ratio of hot to cold forms (K') is plotted against the reciprocal of absolute temperature. The intercept at log $K' = 0.0$ gives the $T_{½}$ for 695 band loss, that at 25 °C permits calculation of $\Delta G_0'$, and $\Delta H_0'$ can be calculated from the slopes (Osheroff *et al.*, 1980).

heme crevice structure in the Aba[78] analogue (Table III) illustrates how crucial to that region is the single hydrogen bond provided by the threonine hydroxyl (Fig. 2). Our data also show that asparagine is not an adequate substitute in this role and suggest that the higher entropic cost of burying the side chain of asparagine, compared to that of threonine, might be the destabilizing factor. Why then is this substitution tolerated in *Chlamydomonas* (Amati *et al.*, 1988)? Fig. 8 shows that at 37 °C this Asn[78] analogue is more than 50% in the unproductive state IV (Dickerson and Timkovitch, 1975) in which the iron-sulfur co-ordination is lost, but that at 10 °C it is >90% in the normal state III. Such a cytochrome would be inefficient in animal species, at an elevated body temperature, but much less so in *Chlamydomonas*, a unicellular alga living in cold water. Alternatively, this organism might possess a change elsewhere in the sequence that can compensate for the threonine to asparagine mutation. That this is feasible is illustrated by the properties of the double, Asn[78], Pro[83], mutant discussed under "Oxidation-Reduction Potential."

Substitution of proline for alanine at position 83 of the vertebrate cytochrome *c* structure causes no spectroscopic changes but does induce a modest change in $\Delta G_0'$ for the temperature- or pH-induced loss of the 695 nm band, and in $\Delta S_0'$ for thermal denaturation (Table III). Since Ala[83] participates in no obvious stabilizing interactions, this weakness is suggestive of a localized conformation change in the heme crevice due to the introduction of the imino acid.

Oxidation-Reduction Potential—In Fig. 10 are collected results of redox potential determinations at pH 7 of all five semisynthetic analogues, and at pH 6 of [Aba[78]]cytochrome *c*. In three cases substantial drops in potential are observed, although that of the Aba[78] analogue is partly restored by lowering the pH to 6. In the native protein the potential is pH-independent in the range 5–8 (Moore *et al.*, 1984).

The potential of a redox center is profoundly influenced by the protein coat that encloses it. Factors that have been proposed to modulate the redox potential of cytochrome *c* are the nature and orientation of the axial ligands (Moore and Williams, 1977), the hydrophobicity of the amino acid side chains immediately packing the heme crevice (Kassner, 1972, 1973), the stability of, and hence solvent accessibility to, the heme crevice (Stellwagen, 1978; Schlauder and Kassner, 1979; Wallace and Proudfoot, 1987), generalized surface charge (Rees, 1980) and special electrostatic interactions (Moore, 1983), as well as the electronic structure of the porphyrin ring as dictated by its substituents (Moore and Williams, 1977; Moore, 1983). It is likely that all these factors play a role, but their relative importance has been a matter of dispute for, until now, it has not been possible to vary these parameters independently within a single system.

In the case of the Phe[67] analogue, for which we observed a potential of 225 mV, compared with 260 mV for the native protein, the axial ligands are unchanged, the heme crevice is no less stable (Table III), and surface charge is unaltered. If the Tyr→Phe transition had significantly increased the global hydrophobicity of the heme pocket, the potential would have risen, rather than fallen (Kassner, 1972, 1973). The remaining possibility is a change in heme electronic structure, so we attribute the variation of potential to the change in electron distribution in the porphyrin induced by the presence or absence of the phenolic hydroxyl group and signaled by the red shift in major absorption bands as discussed above. The potential drop of 35 mV would be sufficiently disruptive to a concerted system of redox carriers like the mitochondrial electron transfer system to be evolutionarily disfavored. For *Euglena* cytochrome *c*, the natural Phe[67]-containing variant, the redox potential is low, although only by about 20 mV (Pettigrew, 1973), so the effect of the change in residue at position 67 may be partially offset by the loss of the thioether link to residue 14 that occurs in this protein. In *Crithidia* cytochrome *c*, also with no thioether, but where residue 67 is tyrosine, a raised potential is observed (Moore *et al.*, 1984). That *Euglena* may be tolerant of this substitution, and consequent change in redox potential, might also be a consequence of a reduced dependence on oxidative phosphorylation. Although a ciliated protozoon, this organism is unusual in possessing chloroplasts.

The analogue in which asparagine replaces threonine at position 78 has a normal redox potential under standard conditions, but the substitution of aminobutyric acid causes a sharp drop (Fig. 10). The almost complete restoration of normalcy in this analogue at pH 6, under which conditions the 695 nm band, and thus the native conformation, is at a maximum, demonstrates that it is the destabilization of the heme crevice that causes the redox potential change, either as a consequence of increased solvent accessibility, or the change in ligation at the sixth coordination position.

The substitution of proline for alanine at position 83 does not significantly affect the redox potential, yet the potential of the double mutant, in which this change is combined with the replacement of threonine by asparagine at position 78 illustrates an important point. That is, in the complex system which constitutes a protein, induced changes are not simply additive and great care must be taken in drawing conclusions about structure-function relations in systems where more than one important residue is affected. In this case, while neither point mutation alone affects redox potential, the combination causes a substantial drop from 260 to 235 mV. Furthermore, the heme crevice of [Asn[78], Pro[83]]cytochrome *c* is more stable than that of the Asn[78] protein, even though the Ala[83] to Pro[83] change is in itself also destabilizing. In terms of resistance to denaturation, the two changes are mutually compensating and thus the synergism of double mutants can operate in either a positive (as in the stabilization of the heme crevice) or negative fashion (as in the establishment of redox potential). These results also strongly support our conclusion that these two properties are independent of one another (Wallace and Corthésy, 1987), and that while the same sorts of interactions stabilize methionine sulfur-iron ligation and redox potential in cytochrome *c*, the set of interactions operative in each case differ.

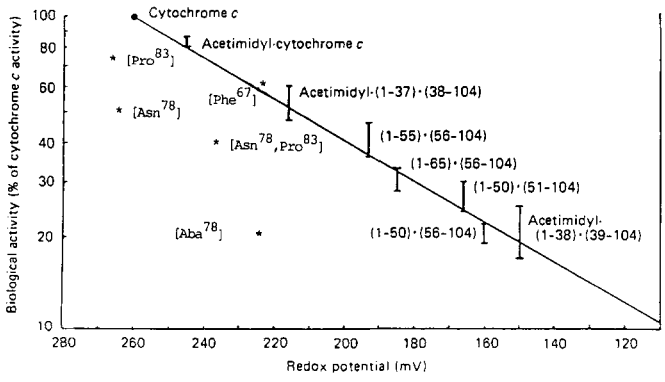

FIG. 12. **The relationship between biological activity and oxidation-reduction potential of semisynthetic cytochromes c.** A set of analogues (*vertical bars*) of varying redox potential have been found previously to show a linear relationship between potential and the logarithm of specific activity. These analogues all involve changes distant from the active site, as do others that obey the relationship. Where residues known to participate in electron transfer are modified, however, the relationship is not obeyed. Those analogues show a more significant drop in biological activity than would be expected should that residue's contribution to maintaining redox potential be its sole influence on electron transfer (Wallace and Proudfoot, 1987). Of the analogues presently tested (*asterisks*) only [Phe^{67}]cytochrome *c* falls on the line, suggesting that both residues 78 and 83 have a direct role in the electron transfer mechanism.

Biological Activities of the Analogues and Implications for the Functional Roles of the Natural Residues at Positions 67, 78, and 83—Fig. 11 shows the oxygen uptake curves recorded in the depleted mitochondria succinate oxidase assay. Only the protein prepared by semisynthesis from the synthetic (66–104) fragment of native sequence is as active as the natural protein. The activities of the analogues are correlated with their redox potentials in Fig. 12.

While the Phe^{67} protein is less active in the succinate oxidase assay than the native protein, its activity is entirely consistent with the diminished redox potential. The relationship that links these two parameters is not obeyed by analogues in which residues forming part of the catalytic site are modified (Wallace and Proudfoot, 1987). Thus the behavior of [Phe^{67}]cytochrome *c* implies that the tyrosine residue is not participating in the catalysis of electron transfer, a role that had been suggested by Takano and Dickerson (1981b).

Both position 78 analogues have biological activities lower than might be expected given their redox potentials. In the Aba^{78} case, this could be explained by an inability of the state IV form (the "hot" or "alkaline" form lacking a 695 nm band) of the protein to accept electrons from reductase (Dickerson and Timkovitch, 1975), despite the relatively high potential observed for this analogue. This implies that the methionine sulfur ligand has a more active role to play in electron transfer than simply establishing the heme potential. In the Asn^{78} analogue, though, both coordination and redox potential are normal. The reduced stability of the heme crevice means, however, that the necessary coordination is less likely to survive the deformation of the active site induced on binding to physiological partners (Weber *et al.*, 1987). Alternatively, or additionally, Thr^{78} may itself play a direct role in electron transfer.

Because oxidation-reduction potential is unaffected in [Pro^{83}]cytochrome *c*, the diminished electron transfer capacity of the analogue shows Ala^{83} to be playing a functional role at the active site of the cytochrome. The analogue thus mimics the properties (normal redox potential but low biological activity) of the higher plant cytochromes (Wallace and Boulter, 1988), which are characterized by the consistent presence of proline at this site. One currently favored view of the mechanism of electron transfer (Poulos and Kraut, 1980; Wendoloski *et al.*, 1987) invokes the movement of phenylalanine 82, on binding of cytochrome *c* to a physiological partner, to a position intermediary to the two redox centers, where its π-electron cloud may provide a channel of high conductance. The rotational restriction imposed, or conformation change induced, by the imino acid at position 83 would impede such a movement of Phe^{82} and result in the observed reduced reactivity. However, our results provide no direct support for this view, since the change that we have induced could, by virtue of the increased bulk of the residue, equally well inhibit any type of active or static matching of complementary surfaces prior to the act of electron transfer. The low activity of the plant cytochromes with rat reductase, that we propose to result at least in part from this same sequence difference, implies that their mode of interaction with the plant reductase differs from the vertebrate model.

Conclusions—The semisynthetic cytochrome *c* prepared with a fully synthetic fragment 66–104 of natural horse sequence is identical with the natural protein in every functional parameter examined. These observations, coupled with the analytical data and the high yields obtained in a stringent conformationally directed religation system, demonstrate that the routine synthesis, by means of highly optimized stepwise solid phase methods, of large peptides of defined covalent structure and high purity is now a practical reality.

We have shown that the natural residue at position 67, tyrosine, is not evolutionarily conserved for a role in structural stabilization. Our data indicate that the hydroxyl group has a specific function in setting the redox potential, probably by influencing electron distribution in the heme and may inhibit autoxidation of ferrocytochrome *c* by blocking access of oxygen. Threonine 78 participates in an important stabilizing interaction. The requirement is so specific that not even asparagine can adequately replace it. The anomalously low biological activity of the analogues prepared suggest that this residue, or methionine 80, has a direct role in electron transfer from physiological partners.

The reduced electron transfer competence of the Pro^{83} analogue mirrors that of the plant cytochromes *c*. Thus Ala^{83}, located in the active site of the protein, is of functional significance and its consistent replacement in plant cytochromes by proline implies that the nature of productive interactions with physiological partners may differ between plant and animal systems.

Semisynthesis of cytochrome *c* using *de novo* synthetic 66–104 is both practical and useful. Its particular value will be in the insertion of noncoded amino acids such as in the very revealing Aba^{78} analogue studied here, or isotopically labeled amino acids at specific locations. This capacity will permit the design of experiments that can help resolve outstanding questions specific to cytochrome function and general to proteins.

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SUPPLEMENTAL MATERIAL TO:

SUBSTITUTIONS ENGINEERED BY CHEMICAL SYNTHESIS AT THREE CONSERVED SITES IN MITOCHONDRIAL CYTOCHROME c: THERMODYNAMIC AND FUNCTIONAL CONSEQUENCES.

BY

Carmichael Wallace, Paolo Mascagni, Brian Chait, James Collawn, Yvonne Paterson, Amanda Proudfoot and Stephen Kent

Experimental Procedures

Materials

Nα-Boc protected -L- amino acids were obtained from the Protein Research Foundation, Osaka, Japan and were used as obtained. Derivatives used were (all Nα -Boc-L-amino acids): Ala, Asn, Ile, Leu, Met, Phe, Pro - side chain unprotected; Lys (2Cl Z); Asp (OBz); Glu (OBz); Thr (Bz); Tyr (BrZ); Arg (N^GTos); and Nα-Boc-Gly. Loaded copoly(styrene-divinylbenzene) Boc-L-Glu(OBz1)-OCH_2 Pam-Resin was obtained from Applied Biosystems, Foster City, California. All chemicals used in peptide synthesis (trifluoroacetic acid, dichloro-methane, dimethylformamide, diisopropylethylamine, dicyclohexylcarbodiimide, and 1-hydroxybenzotnazole) and in the quantitative ninhydrin determination of uncoupled amine (Sarin, et al., 1981) were obtained from Applied Biosystems. Anhydrous hydrogen fluoride was obtained from Matheson. Cytochrome c (type III) was from Sigma. All other chemicals were AR grade or better and were obtained from Aldrich Chemical Company, Milwaukee, Wisconsin.

Methods

Peptide Synthesis

Peptides were prepared by stepwise solid phase synthesis (Merrifield, 1963; Kent, 1988) on a commercially available peptide synthesizer (Kent, et al., 1984; Kent, et al., 1985) modified by the removal of in-line filters to the top and bottom of the reaction vessel. A 7 ml Teflon-Kel F reaction vessel was used (designed by Steven Clark, machined by Frank Ostrander, Biology Division, California Institute of Technology). Rapid synthetic protocols, developed at Caltech (Kent et al., 1988), were used. Synthesis was initiated from the carboxyl terminal L-Glu (OBzl) residue attached to copoly(styrene-divinyl benzene) resin (loading: 1 mmol amino acid per gram of resin), on a 0.1 mmole scale. The following steps were used for the addition of each amino acid residue to the polypeptide chain:

1. Deprotection: The NαBoc group was removed by a 4 minute treatment with neat (100%) TFA. The bulk of the TFA was removed by filtration under nitrogen.
2. Neutralization: After a single continuous flow wash with DMF (45 sec.), the salt of the peptide-resin was neutralized by treatment with 10% v/v DIEA in DMF (2xl min).
3. Peptide bond formation: After a single prolonged continuous flow wash with DMF (90 sec.), excess solvent was removed by filtration. The activated Boc-amino acid solution (0.5 mmol symmetric anhydride; 1 mmole HOBt ester) was introduced in a minimal volume of DMF (3-4ml total volume; 0.15-0.3 M activated amino acid). After 10 minutes reaction (20 minutes, HOBT esters), excess reagents and coproducts were removed by a continuous flow wash with DMF (1 minute), and a sample (5mg) of peptide-resin taken under instrument control and deposited in a fraction collector, for documentation of the efficiency of peptide bond formation by means of the quantitative ninhydrin determination of residual amine (Sarin et al., 1981). Finally the excess DMF was removed (prior to the TFA removal of the NαBOC in the next cycle of synthesis) by filtration.

Activated Boc-amino acids were formed on the instrument immediately prior to use, as previously described (Kent et al., 1984, 1985; Kent 1988). Only a single peptide bond - forming step was performed for each residue. Protected NαBoc-peptide resins were stored at 4°C.

Immediately prior to deprotection/cleavage, the NαBoc group was removed by TFA treatment and the neutralized peptide-resin thoroughly dried after washing with DCM. Peptides were cleaved from the resin and the side-chain protecting groups removed at the same time, by treatment with HF using a modified S_N2-S_N1 protocol (Tam, et al., 1983), in a Kel F cleavage apparatus (Tojo, Japan), as follows: after removal of the Boc group, the neutralized peptide - resin (300 mg) was stirred in dimethylsulfide (6.5 mL) - p-cresol (0.8 mL) -p-thiocresol (0.2 mL) for 3 min., then HF was condensed as a liquid (2.5 mL) and the peptide-resin suspension was stirred for 90 minutes at 0°C. The partly-deprotected peptide-resin was recovered by pouring the suspension through a PTFE filter on a nalgene funnel (**Care: HF is an extraordinarily toxic and corrosive compound**), and was thoroughly washed with dichloromethane and dried under aspirator vacuum. The remaining protecting groups were removed and the peptide cleaved from the resin by treatment with HF (9 mL)/p-cresol(0.4 mL)/p-thiocresol (0.1 mL) for 1 hour at 0°C. After the second (high concentration of HF, S_N1) step, the bulk of the HF was removed under water-aspirator vacuum, and the peptide products were precipitated by the addition of diethyl ether. Excess residual scavenger(s) was removed by thorough trituration with ether, and the total crude peptide products were dissolved in acetic acid-water (80:20, v/v) and lyophilized.

Purification was performed by semi-preparative reverse phase HPLC on a Vydac C4 column (1 x 30 cm; 5 μ particle size), using a gradient of 0 - 60% CH_3CN vs. 0.1% TFA, developed over 300 minutes at a flow rate of 3.0 ml/min (Clark-Lewis and Kent, 1989). Fractions containing purified peptide were identified by rapid analytical HPLC of aliquots, and the appropriate fractions were combined and lyophilized. The purified peptides were characterized by analytical reverse phase HPLC (Vydac C4, 0.46 x 30 cm), by amino acid analysis, and by ^{252}Cf plasma desorption time-of-flight mass spectrometry (Chait, 1987, 1988).

Semisynthesis of cytochrome c analogues

Synthetic fragments were combined with the homoserine lactone form of CNBr fragment 1-65, by exploiting the spontaneous aminolysis of the lactone, a mildly activated ester, by the free α-amino group of 66-104. Natural 1-65 was prepared from cytochrome c by CNBr cleavage and gel filtration according to Corradin and Harbury (1970). This product was purified by cation-exchange chromatography as described by Wallace and Proudfoot (1987), which also resolves the lactone and open-ring forms of the fragment. The conditions for fragment condensation were as described by Wallace and Rose (1983).

Purification of cytochrome c analogues

Cation-exchange chromatography of ferricytochromes c in non-denaturing conditions is effective in separating coupled products from impurities, principally deamidated forms and fragment polymers (Wallace and Harris, 1984). In order to rid the products of any of these latter that might fortuitously co-elute with ferricytochromes c, we chromatographed the proteins a second time on the same system, but in the reduced form. The change in charge and molecular dimension on reduction leads to a substantial shift in the elution position. The purity of the products was checked by UV-visible spectroscopy and by ^{252}Cf plasma-desorption time-of-flight mass spectrometry (Chait, 1987).

Spectral characteristics of the analogues

The absorbance spectra in the range 750-250nm were recorded on a Beckman model 35 spectrophotometer. Any deviation from the characteristic absorbance maxima and extinction coefficients of the native protein can give information on both the structural and electronic state of the cytochrome.

Titration of the 695nm band

pH titrations were performed as described by Wallace (1984), and thermal titrations were done in a Beckman DU-8 spectrophotometer equipped with a T_m module. The instrument was programmed to gather absorbance values at 2° intervals over the range 0-90°. Cytochrome analogue samples were dissolved in the sodium cacodylate/sodium chloride buffer of Osheroff et al (1980). Most thermal titrations could not be taken to completion, due to protein precipitation. In these cases the absorbance due to neighboring bands at 695 nm after complete elimination of that due to the Fe-S bond was taken to be that observed in pH titrations, an assumption that was seen to be true in those cases where the thermal titration was completed before precipitation onset.

The end-point of the titration, either determined or extrapolated, was used to calculate log K' for the transition. Arrhenius plots of this value versus the reciprocal of absolute temperature allows calculation of the thermodynamic parameters for the opening of the haem crevice.

Oxidation-reduction potentials of the analogues

Redox potentials at pH 7 were determined by the method of Wallace et al (1986). In one case a potential was also determined at pH 6, after adjustment of the redox buffer with concentrated HCl. We use an adaption of the method of mixtures, in which the proportions of the components of the ferro/ferricyanide redox couple (of known E_m, 430mV) in the cytochrome-containing buffer are varied. A measurement of A_{550}, with reference to the absorptions of the fully oxidised and reduced proteins, allows calculation of the proportions of the components of the ferro/ferricytochrome c couple, of unknown E_m. The logarithms of these two ratios are plotted as in figure 10. A slope of unity is expected of a single-electron transfer and the mid-point potential for the cytochrome can be calculated, using the Nernst equation, from the proportions of ferro and ferricyanide corresponding to a ferro/ferricytochrome ratio of 1.

Biological assays

The efficiency of the analogues in restoring O_2 uptake to cytochrome c-depleted mitochondria in a succinate oxidase assay was compared to that of the native protein (Wallace and Proudfoot, 1987).

Radioimmunoassay with monoclonal antibodies to native cytochrome c

Anti-horse cytochrome c antibodies C3, C7, E3 and E8 were produced and purified as previously described (Carbone and Paterson, 1985). Assays were performed on microtiter plates according to Collawn et al (1988) at various dilutions of affinity purified antibody solution. The undiluted concentrations of antibodies in mg ml^{-1} were 0.48 (C3), 1.43 (C7), 0.50 (E3) and 0.73 (E8).

Results and Discussion

Peptide Synthesis

The target sequences shown in Figure 1 were prepared in side-chain protected form by step wise solid phase peptide synthesis (Merrifield, 1963). All reactive side chain functionalities were protected by groups stable to the conditions of chain assembly, but removable by treatment with HF with minimal damage to the product peptide. The Boc group was used to protect the α-amino function of the activated amino acid during the formation of each peptide bond. Highly optimized synthetic protocols were used in instrument-assisted chain assembly (Kent, 1988).

Key features of the improved synthetic protocols included the use of a loaded Boc-amino acyl- 4 (carboxamidomethyl) benzylester-resin stable to the conditions of chain assembly, prepared in a form free of undesired functional groups by an unambiguous route (Mitchell et al., 1978). In a departure from previous practice, dichloromethane, a poor solvent for the peptide-resin, was eliminated from the synthetic protocols. Thus, neat (100%) TFA was used to remove the NαBoc group at each cycle of synthesis. Studies have shown this to rapidly (< 4 minutes) effect complete removal of the NαBoc group at all stages of a synthesis (S.B.H. Kent, unpublished results). Considerations of the nature of diffusion between the smaller peptide-resin matrix and surrounding solvent led us to adopt more efficient, rapid "flow washing" protocols throughout the chain assembly. A single 30 sec. flow wash with dimethylformamide, a good solvent for peptide-resins, has been shown to be the equivalent of four 1 minute batchwise washes (S.B.H. Kent, unpublished results). The peptide resin was filtered at only three points, to minimize the carry over of residual solvent, in the extension of the resin-bound peptide by each amino acid. Formation of the peptide bond was accomplished by reaction of the activated Boc-amino acid with the neutralized peptide-resin in DMF. Only a minimal volume of solvent was used in the coupling reaction, resulting in extremely fast reaction and high yields.

Synthetic protocols based on these principles were extremely rapid. The rate of synthesis was approximately 22 minutes per amino acid, allowing the 39 residue peptide chains to each be assembled overnight (14 hours). Average chain assembly yields were 99.6% per residue, as measured by the quantitative ninhydrin reaction, corresponding to an 86% overall yield of the target protected peptide sequence. The coupling of Ile^{95} was reproducibly poor in all syntheses, giving a yield of only 96%. The reason for this is not known, but may be due to slower reaction of the β-substituted amino acid, perhaps combined with sequence-dependent aggregation of the (96-104) peptide resin (Live and Kent, 1984). Synthesis was started with 0.1 mmol Boc-Glu (OBz1) OCH_2 Pam-resin, and gave about 300 mg (0.04 mmol) Boc-(66-104)-resin product (yield not corrected for 38 peptide-resin samples taken during the synthesis).

Treatment with HF in the presence of scavengers was used to remove all sidechain protecting groups, and cleave the peptide-resin bond to release the free peptide. The NαBoc group was first removed with TFA, to prevent tert-butylation of the Met^{80} side chain (Noble et al., 1976).

Deprotection/cleavage by a standard ("high") HF protocol (HF/p-cresol/p-thiocresol; 90:5:5 v/v, 1hr. 0°C) gave a large number of byproducts when the total crude material was examined by analytical reverse-phase HPLC. These byproducts eluted later than the free peptide and showed an enhanced $OD_{254/214\ nm}$ ratio. Furthermore, under prolonged heating at 80°C in aqueous solution, these byproducts were partly converted to the target peptide. Together, these data suggested that the byproducts arose from alkylation of Met^{80}, Tyr^{67}, Tyr^{74} and Tyr^{97} by reactive aromatic species (benzyl carbonium ions) arising during the HF cleavage. The unusually large amount of such byproducts was probably due to the very high content of Tyr (three residues) and Lys (2ClZ) (eight residues) in the target sequence.

A modified version of the S_N2-S_N1 HF deprotection procedure (Tam et al., 1983) was used to overcome this problem. In this way, very high yields of remarkably pure crude products were obtained (Figure 3 (a)). Typically, the target peptide constituted about 80 mol % of the total crude products when analyzed by reverse phase HPLC. Cleavage of 300 mg of peptide-resin typically gave 170 mg of lyophilized product. Amino acid analyses after acid hydrolysis showed that this crude product was about 70% by weight peptide, with the remainder being associated salts and water. Purification of 70 mg amounts of crude products by semi-preparative reverse phase HPLC (Vydac C4, 1 x 30 cm) gave 15-25 mg of highly purified peptide after lyophilization of the fractions identified by rapid analytical HPLC. These products were remarkably clean when examined by analytical HPLC (Figure 3(b)).

Purified peptides were characterized by amino acid analysis after 6N HCl hydrolysis and gave the expected ratios, within experimental error (data not shown). A much more stringent analysis of the covalent structures of some of the synthetic 39 residue peptides was performed by ^{252}Cf plasma desorption time-of-flight mass spectrometry (Chait, 1988). This technique gives mass spectra of peptides with relatively intense peaks corresponding to the protonated intact peptides. Quite accurate mass measurements are possible, and this accurate mass measurement serves as a rigorous check on the accuracy of the proposed covalent structure of a synthetic peptide. A typical mass spectrum is shown in Figure 3(c). Measured masses of the synthetic peptides are in excellent agreement with the molecular masses calculated from the target sequences, confirming the covalent structures and demonstrating the absence of unexpected covalent modifications

The "purified" product of the initial synthesis of the Asn^{78} (66-104) sequence coupled poorly in the religation with 1-65 Hse lactone. Subsequent mass spectral examination showed the presence of about 25 mol % of a peptide with an Asn deleted from the target sequence. Re-

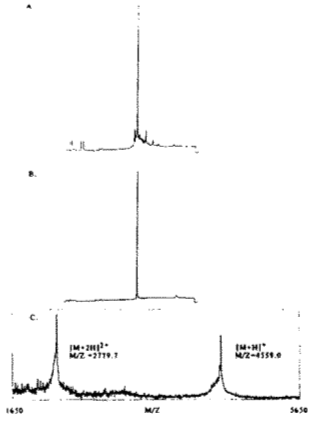

Figure 3. Characterization of a synthetic 66-104 fragment (Phe^{67} analogue) by HPLC and ^{252}Cf plasma-desorption mass spectrometry.

Panel A: HPLC analysis of crude product cleaved from the resin by the low-high HF method described in the text. Panel B: HPLC analysis of pooled fractions of major peak from preparative HPLC purification of crude product. HPLC was performed as described in the text. Panel C: Mass spectrum showing the singly and doubly protonated molecular ions. The molecular mass determined for this peptide is 4557.7, that calculated from the sequence is 4557.4.

examination by analytical C18 reverse phase HPLC revealed the same impurity, which had co-chromatographed on the C4 HPLC separation. Detailed enzyme digestion/mass spec. studies showed that the contaminant was [des-Asn^{103}] (66-104) (B.Chait, unpublished results). Re-examination of the ninhydrin data in the synthetic records showed no indication of a problem in coupling at that position. The synthetic error was therefore due to a failure to remove about 20% of the N$^{\alpha}$Boc group from the preceeding residue. Resynthesis of the Asn^{78} sequence gave a high yield of the correct product, with a molecular mass of 4586.3 determined by time-of-flight mass spectrometry. The calculated mass is 4586.4. There were no detectable modified or error sequences in this or any of the other synthetic products used for the studies reported in this paper.

TABLE I

Coupling yields and experimentally-determined masses of semisynthetic cytochrome c analogues resynthesised from equimolar mixtures of fragment 1-65 and natural and synthetic 66-104.

Fragment	Yield	Molecular Weight	
Naturally obtained 66-104	up to 60%	Observed	Expected
Synthetic 66-104 of native sequence	58%	12335	12330
[Phe^{67}] 66-104	56%	12313	12314
[Pro^{83}] 66-104	53%	12381	12356
[Asn^{78}, Pro^{83}] 66-104	52%	12383	12369
[Aba^{78}] 66-104	33%	12345	12314

Yields were estimated by comparing peak heights of coupled products on the gel-filtration elution profile with those of unreacted fragments; molecular weights were obtained by ^{252}Cf plasma desorption time-of-flight mass spectrometry. Expected molecular weights were calculated by adjustment of the value for the native protein by the introduced changes: i.e. that included in the 66-104 fragment and the replacement of methionine by homoserine at position 65 as a consequence of the religation method. The value for the native protein given in Margoliash (1962) and propagated in the literature (and on the Sigma bottle) of 12,384 is in error. The correct value is 12,360.

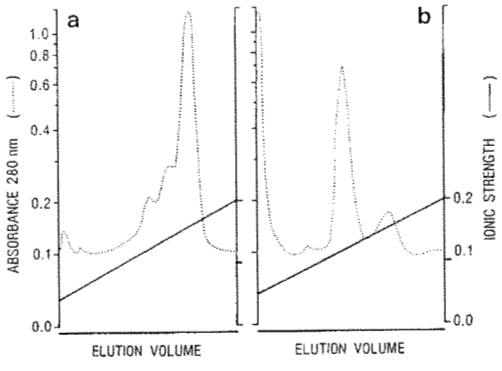

Figure 4. Elution profiles of ion exchange chromatography of crude [Phe^{67}] cytochrome c.

In (a) the product eluting at the cytochrome c position on Sephadex G50 gel-filtration of coupling mixtures is applied in the ferric form to a 1cm x 10cm column of Trisacryl SP cation-exchanger, equilibrated in 0.04 M potassium phosphate buffer, pH 7.0 and eluted with a linear gradient formed from that buffer and 0.2 M potassium phosphate, pH 7.0. In (b) the major peak from (a) is reapplied after reduction by ascorbate, and eluted with the same gradient.

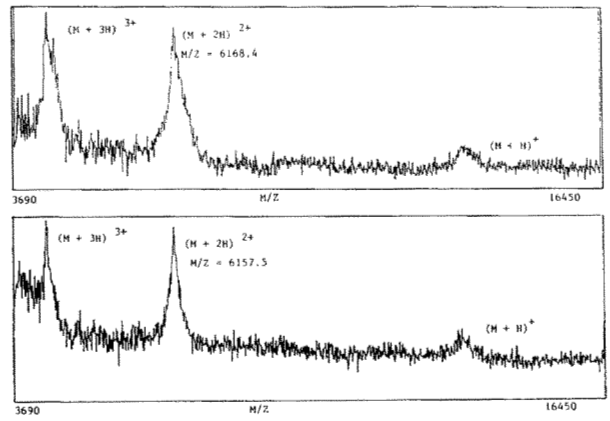

Figure 5. Plasma desorption time-of-flight mass spectra of cytochrome c analogues.

Illustrated are the spectra obtained in the M/Z range 3690-16450 for two semi-synthetic products, of natural sequence (upper figure) and the Phe67 analogue (lower). In each case only peaks corresponding to the tripely, doubly and singly proto ated molecular ions of the expected product are observed.

TABLE II

Spectral properties of cytochrome c analogues at pH 7 and 25°

	Native Horse	[Phe^{67}]	[N^{78},P^{83}]	[Pro^{83}]	[Aba^{78}]	[Asn^{78}]
695 nm band	Present	Present	Present	Present	Weak	Not fully developed
Ferro α band (nm)	550	553	550	550	550	550
Ferro β band (nm)	520	523	520	520	520	520
Ferri α band (nm)	528	531	528	528	528	528
Ferro 550/ 535 nm	3.4	2.9	3.5	3.4	3.5	3.6
360 nm band	Present	Present	Present	Present	350 nm	Present
Ferri 280/ 360 nm	0.82	0.79	0.82	0.82	0.76	0.82

The 695 band is discussed in the text, the α and β bands are characteristic of c-type cytochromes and are normally unvarying.

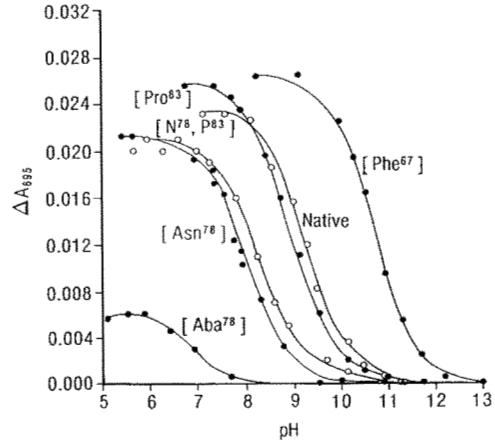

Figure 7. Plots of the change in absorbance at 695 nm with increasing pH for cytochrome c and its analogues.

Samples were dissolved in 0.05 M potassium phosphate buffer at 24° and the pH raised by addition of 1 M KOH. The pKs for the alkaline transition of each analogue determined from these curves are shown in table 3.

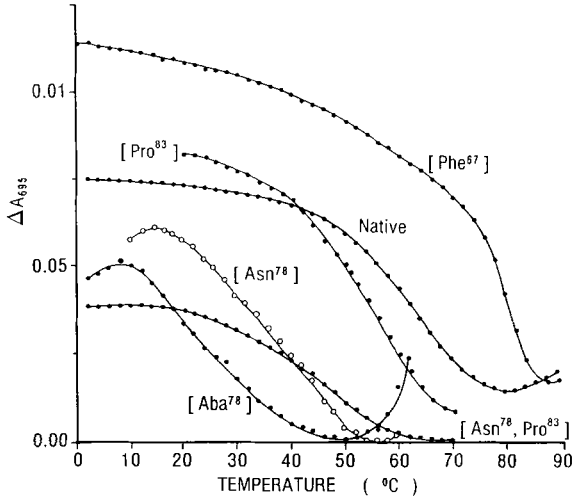

Figure 8. Plots of the change in absorbance at 695 nm with increasing temperature for cytochrome c and its analogues.

Sample solutions were made up in 10 mm sodium cacodylate, 10 mm sodium chloride, pH 7. Absorbance measurements were made at 2° intervals over all or part of the range 0-90°. These data were used to construct the Arrhenius plots shown in Figure 5 from which the $T_{1/2}$ and thermodynamic parameters shown in Table 3 were calculated.

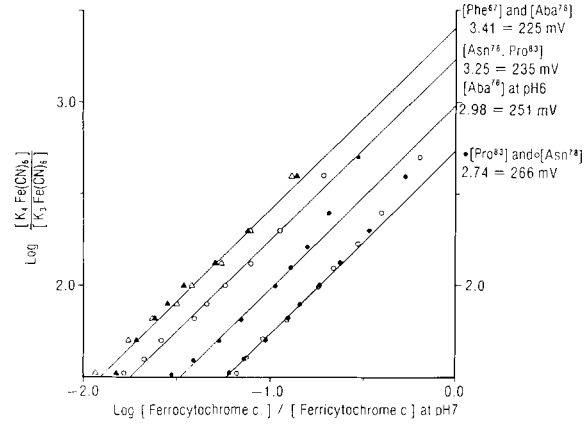

Figure 10. Redox titrations of analogues of cytochrome c at pH 7

Also included is a titration of the Aba78 analogue at pH 6. Values for the midpoint oxidation-reduction potentials are calculated from the ferro/ferricyanide ratio obtained by extrapolation to the mid point of cytochrome reduction, as described by Wallace et al. (1986). Values of 260 mV were obtained for native horse cytochrome c and the semisynthetic product of natural sequence (data not shown).

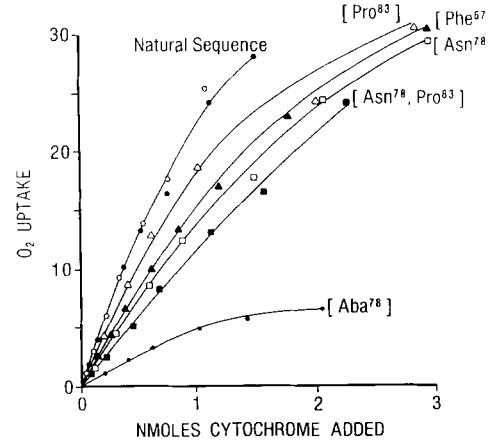

Figure 11. Biological activity curves for cytochrome c and its analogues.

Sequential additions of cytochrome are made to the chamber of the O_2 electrode. The succinate oxidase activity of cytochrome c - depleted mitrochondia is followed by polarographic measurement of oxygen consumption (as % total dissolved O_2 consumed per minute). At limiting cytochrome c concentrations the system assays the specific activity of cytochromes c with the reductase (complex III) (Wallace and Proudfoot, 1987). For measurements of the specific activity of the protein of natural sequence, both native (●) and semisynthetic (○) proteins were used.