# Conversion of a *c* type cytochrome to a *b* type that spontaneously forms *in vitro* from apo protein and heme: Implications for *c* type cytochrome biogenesis and folding

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Communicated by Harry B. Gray, California Institute of Technology, Pasadena, CA, March 1, 2000 (received for review December 27, 1999)

**Cytochrome $c_{552}$ from *Hydrogenobacter thermophilus*, a thermophilic bacterium, has been converted into a *b* type cytochrome, after mutagenesis of both heme-binding cysteines to alanine and expression in the cytoplasm of *Escherichia coli*. The *b* type variant is less stable, with the guanidine hydrochloride unfolding midpoint occurring at a concentration 2 M lower than for the wild-type protein. The reduction potential is 75 mV lower than that of the recombinant wild-type protein. The heme can be removed from the *b* type variant, thus generating an apo protein that has, according to circular dichroism spectroscopy, an $\alpha$-helical content different from that of the holo *b* type protein. The latter is readily reformed *in vitro* by addition of heme to the apo protein. This reforming suggests that previously observed assembly of cytochrome $c_{552}$, which has the typical class I cytochrome *c* fold, in the *E. coli* cytoplasm is a consequence of spontaneous thioether bond formation after binding of heme to a prefolded polypeptide. These observations have implications for the general problem of *c* type cytochrome biogenesis.**

The *c* type cytochromes are a class of electron transport proteins characterized by the covalent attachment of a heme moiety to a peptide via thioether bonds between the vinyl groups of the heme and the cysteinyl thiols of the CXXCH binding motif(s) of the protein (1). A few protozoan mitochondrial *c* type cytochromes, c (2, 3) and $c_1$ (4), have heme attached through a single thioether bond. In all these known examples, it is the first cysteine of the binding motif that is lost, with heme attachment to the second. These examples are consistent with expression in yeast of human mitochondrial cytochrome *c*, which has shown that C14, but not C17, can be removed (5).

Assembly of holo *c* type cytochromes requires the action of other proteins: either a cytochrome *c* heme lyase, as in yeast and mammalian mitochondria, or components of one of the more complex systems, requiring of the order of 10 gene products as found in bacteria, chloroplasts, and plant mitochondria (6, 7). The advantages of forming *c* type cytochromes are not fully apparent (8).

Bacterial *c* type cytochromes are all either membrane anchored, with their heme-binding domains on the external side of the membrane, or, in the case of Gram-negative organisms, in the periplasm. Expression of heterologous *c* type cytochromes in *Escherichia coli* generally requires targeting of the protein to the periplasm for assembly by the *c* type cytochrome biosynthesis machinery (9). There are currently three known exceptions. One is that yeast mitochondrial cytochrome *c* can be synthesized in the *E. coli* cytoplasm but only if there is coproduction of the yeast cytochrome *c* heme lyase (10). The other two exceptions both involve highly thermostable bacterial cytochromes *c*. Cytochrome $c_{552}$ from *Thermus thermophilus* has been produced in the *E. coli* cytoplasm but yielded several spectrally distinct proteins, one of which is identical to the authentic holo protein (11, 12). *Hydrogenobacter thermophilus* $c_{552}$ forms holo protein, which is indistinguishable from the native protein by visible absorption spectroscopy, in the cytoplasm of *E. coli* (9). The CD spectra of the recombinant and native forms are also the same, but it has been shown that the presence of the N-terminal methionine in the cytoplasmically expressed material does reduce slightly the thermostability relative to the native protein or recombinant protein isolated from the periplasm of *Pseudomonas aeruginosa* (13).

Some heme proteins retain an essentially unaltered tertiary structure after removal of heme. One example is the periplasmic cytochrome $b_{562}$ from *E. coli* for which it is possible to reform the holo cytochrome $b_{562}$ by mixing the apo protein with heme (14). This behavior has not been observed for any *c* type cytochrome. The most widely studied *c* type cytochrome is mitochondrial cytochrome *c*, which, in its holo form, has a highly $\alpha$-helical structure (15). Removal of the heme results in an apo form that has a random coil structure, as judged by several techniques, including CD (16, 17). Heme has never been shown to attach covalently *in vitro* to this unfolded apo form. There are, however, reports of apo mitochondrial cytochrome *c* adopting a more compact structure in the presence of heme (18) and a mainly helical structure in the presence of some lipids (19).

Many species of bacteria contain one or more low molecular weight mono-heme *c* type cytochromes that share a similar class 1 cytochrome *c* structural fold with the mitochondrial bacteria proteins (20). *H. thermophilus* cytochrome $c_{552}$ is an example of this class of proteins as was shown by the three-dimensional solution structure (21). Little is known about the apo forms of these bacterial proteins, but the requirement of a complex biosynthetic apparatus (6, 7) suggests that the attachment of heme may be significantly more complex than the binding of the heme to a preformed pocket as is thought to happen with both cytochrome $b_{562}$ and $b_5$ (22).

The above considerations imply that attachment of heme to a CXXCH sequence is not usually a spontaneous process. However, it has been suggested that the very high thermal stability of holo cytochrome $c_{552}$ means that, unusually, the apo protein has a tertiary structure with a pocket that may bind the heme in the correct orientation to allow spontaneous formation of the thioether bonds (9). This attachment could readily occur in the reducing environment of the cytoplasm, where the two cysteine residues should not form a disulfide. Holo cytochrome $c_{552}$ formation in the cytoplasm does not, accordingly, require the normal biosynthesis machinery (23). The aim of the present work has been to investigate this proposal further by mutating the cysteine residues responsible for forming the thioether bonds in *H. thermophilus* cytochrome $c_{552}$.

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Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.090089397. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.090089397

If the apo protein does have a stable structure, replacement of both cysteines by alanine might be expected to generate a *b* type cytochrome with noncovalently bound heme.

## Materials and Methods

All experiments were carried out with *E. coli* strain XL1-Blue from Stratagene. Cultures for protein expression were grown in 2TY medium (16 $gl^{-1}$ bacteriological peptone/10 $gl^{-1}$ yeast extract/5 $gl^{-1}$ sodium chloride) in 2.5-liter flasks. Cultures were grown at 37°C in an orbital incubator with shaking at 200 rpm. All other cultures were grown in LB. DNA was prepared with Promega Wizard minipreps.

Oligonucleotides were synthesized by PE-Applied Biosystems and supplied at ReadyPure specification.

**Mutagenesis.** Mutagenesis was carried out by PCR by first using pKHC12, which contains the cytochrome $c_{552}$ gene without a periplasmic targeting sequence (9), as a template and oligonucleotides EJT3 5′-GCATGAATTCATGAATGAACAGCTTGCCAAGCAAAAGGGCGCCATGGCTTGCCACGATCT-3′ and EJT5 5′-CGTCAGAAGCTTTTACTTTATGGAGAGTATCC-3′. The resulting 250-bp fragment was digested with *Eco*RI and *Hin*dIII and cloned into the Amersham Pharmacia expression vector pKK223-3 to yield pEST201, which had an introduced *Nco*I site at the first mutation site. Further PCR with pEST201 as the template and oligonucleotides EJT4 5′-GCGCCATGGCTGCACACGATCTGAAAGCTAAGAAGG-3′ and EJT5 gave a fragment that was digested with *Nco*I and *Hin*dIII and cloned into pEST201. This procedure generated pEST202, which contained mutations of the only two cysteines to alanines. Both plasmids were sequenced to ensure that only the required mutations were present.

**Expression and Purification of Cytochromes.** An overnight culture of *E. coli* XL1-Blue (6 ml) containing pKHC12 or pEST202 in 2TY (16 $gl^{-1}$ tryptone/10 $gl^{-1}$ yeast extract/5 $gl^{-1}$ sodium chloride/100 $mg{\cdot}ml^{-1}$ ampicillin) was used to inoculate 600 ml of 2TY with 100 $mg{\cdot}ml^{-1}$ ampicillin in a 2.5-liter flask. When the optical density of the culture at 550 nm had reached 0.8, isopropyl β-D-thiogalactoside was added to a final concentration of 1 mM, and the culture was incubated for a further 6 h. Cells were harvested by centrifugation. Expressed cytochrome was purified as described (24) with ion exchange chromatography on CM52 Sepharose followed by gel filtration with G50 Sephadex. C11A/C14A holo protein was expressed at levels approximately four times greater than those of the wild-type protein. The N-terminal amino acid sequences up to residue 16 were determined for each protein and shown to be correct. Protein concentrations were determined by using the method of Bradford and the theoretical extinction coefficient at 280 nm.

**Determination of Reduction Potentials.** These were measured by equilibrium potentiometry as described by Dutton (25).

**Retention of Heme by C11A/C14A.** The retention of heme by C11A/C14A was analyzed by dialysis of the air-oxidized protein against 1,000 volumes of sodium phosphate buffer (pH 7.3) at 4°C. Periodically, a sample was removed from the dialysis tubing, and the visible absorption spectrum was measured from 280–600 nm. The sample was then reduced by the addition of a few grains of sodium dithionite; the spectrum was recorded again and compared with the spectrum of the sample before dialysis. The ratio of heme content to the amount of protein was monitored by using the ratio of $A_{425}$ of dithionite reduced protein:$A_{280}$ of oxidized protein.

**Preparation of Apo C11A/C14A.** Two methods were used. (*i*) Acid butanone extraction of heme was carried out according to the method of Teale (26), followed by dialysis first against 1,000 volumes of 100 mM sodium phosphate (pH 7.3), then against two sets of 1,000 volumes of 20 mM sodium phosphate (pH 7.3). (*ii*) A 0.4 mM solution of purified holo C11A/C14A in 20 mM sodium phosphate buffer (pH 7.3) containing 1 M imidazole was incubated overnight at 4°C. The red precipitate was removed by centrifugation, and the clear supernatant was dialyzed against 1,000 volumes of 20 mM sodium phosphate buffer (pH 7.3). Apo C11A/C14A was purified from the residual holo protein by using CM52 Sepharose and a 0–0.5 M sodium chloride gradient.

**Reconstitution of Apo Protein.** Reconstitution of apo protein with hemin was achieved by addition of 1-μl aliquots of a 1 mM solution of hemin (Sigma) in DMSO to 1 ml of 9 μM protein in 20 mM sodium phosphate buffer (pH 7.3) at 25°C. The DMSO prevents aggregation of the heme, which greatly reduces the rate of reconstitution of hemoproteins (27). Samples were mixed by inversion, and spectra were recorded immediately. For reconstitution under reducing conditions, a few grains of sodium dithionite were added to the protein solution before heme addition.

Visible spectra were recorded by using a Perkin–Elmer Lambda 2 UV/VIS spectrophotometer. Pyridine hemochrome spectra were obtained according to the method of Bartsch (28). CD spectra were taken with a Jasco J720 spectropolarimeter with a fused quartz cuvette of 1-mm path length and protein concentrations of 25–30 μM. All spectra were recorded at 25°C unless otherwise stated.

All thermal denaturation experiments were carried out with a ramp rate of 30°C/h.

**Unfolding of Proteins in Guanidine Hydrochloride.** Unfolding of proteins in guanidine hydrochloride was carried out by appropriate dilution of an 8 M stock solution before addition to concentrated protein solution, yielding a final protein concentration of 30 μM in 20 mM sodium phosphate (pH 7.3). Samples were equilibrated at 25°C for 2 h before spectra were measured. Unfolding of the C11A/C14A protein was monitored by the decrease in the Soret absorption at 416 nm for the oxidized form and at 425 nm for the reduced form. Wild-type protein unfolding was monitored by the change in 419 nm for the oxidized form. The latter changes in absorbance were obtained from difference spectra with protein in buffer alone as the reference. This procedure facilitates following the unfolding of *c* type cytochromes, because, due to the covalent attachment of the heme to the polypeptide, only the methionine ligand is lost, resulting in a red-shifted Soret band of increased intensity (29), rather than a loss of the Soret as for *b* types. All solutions for unfolding studies under reduced conditions were degassed and, together with the cuvettes, flushed with argon.

**Determination of Stability to pH.** Determination of stability to pH was carried out by diluting a concentrated protein stock solution with a solution containing the following buffers to a final concentration of 10 mM each: acetate, Hepes, Mes, Caps, Ches, and Tris·HCl. The pH was adjusted with hydrochloric acid or sodium hydroxide as appropriate. Changes in the Soret region were monitored at 416 nm for the C11A/C14A protein and 410 nm for the wild-type cytochrome $c_{552}$.

## Results

**Characterization of C11A/C14A Protein.** Expression from pEST202, carrying the C11A/C14A mutations, gave a red protein with the characteristics of a *b* type cytochrome. The key features of the visible absorption and pyridine hemochrome spectra of the wild-type and C11A/C14A proteins are given in Table 1. The pyridine hemochrome α-absorption maximum of 556.5 nm for the C11A/C14A protein is consistent with the presence of a *b* type heme. Table 1 also shows that the loss of the two thioether bonds resulted in the midpoint reduction potential decreasing by

**Table 1. Comparison of reduction potentials and visible absorption spectra, for oxidized, reduced, and pyridine hemochrome forms, of cytochrome $c_{552}$ and the C11A/C14A mutant**

| Protein | $\lambda_{max}$, nm | | | Reduction potential, E°′/mV |
|---|---|---|---|---|
| | Oxidized | Reduced | Pyridine hemochrome | |
| Wild type | 410 | 417 | 550 | 245 |
| | 529 | 521 | | |
| | | 552 | | |
| C11A/C14A | 416 | 425 | 556.5 | 170 |
| | 536 | 529 | | |
| | | 560 | | |

E°′, standard reduction potential at pH 7.

75 mV. Fig. 1 provides a comparison of the visible absorption spectra of the wild-type and C11A/C14A proteins, each in the reduced state.

**Stability of Heme Attachment to C11A/C14A Protein.** The disadvantage of irretrievable heme loss from *b* type cytochromes in the periplasm has been suggested as a reason for the evolution of covalent attachment of heme in *c* type cytochromes (30). The strength of heme binding to C11A/C14A was investigated by dialyzing a 1 mg·ml$^{-1}$ protein solution against 1,000 volumes of buffer and monitoring changes in the ratio of absorbance at 425 nm (reduced protein), which reflects the heme content, versus 280 nm (oxidized protein), which indicates the protein content. The absorption of dithionite at 280 nm obscures the contribution of the reduced protein to the absorbance at this wavelength. Over 5 days of dialysis, less than 15% of the heme was lost from the protein. It was shown separately that heme could pass readily through the dialysis membrane. There was no change in the spectra of the reduced or oxidized forms of the C11A/C14A protein after 5 days, indicating no damage to the heme environment within the protein.

**Formation of C11A/C14A Apo Protein.** Acid butanone extraction has been used successfully to generate a number of apo forms of hemoproteins, for example apo cytochrome $b_{562}$, which retains its tertiary structure and can be readily reconstituted with free heme (14). This method was effective in removing heme from C11A/C14A protein, as judged by the visible absorption spectrum, which was featureless above 300 nm. This result confirms that heme was noncovalently bound in this protein. Addition of free heme in either the reduced or the oxidized state to this apo protein resulted in a visible spectrum resembling that of free heme rather than that of a cytochrome. Far UV CD spectroscopy

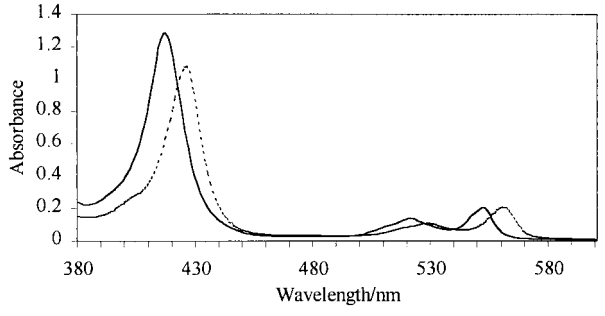


**Fig. 1.** Comparison of the visible absorption spectra of 15 $\mu$M reduced wild-type (solid line) and C11A/C14A (dashed line) cytochrome $c_{552}$ in 20 mM sodium phosphate (pH 7.3).

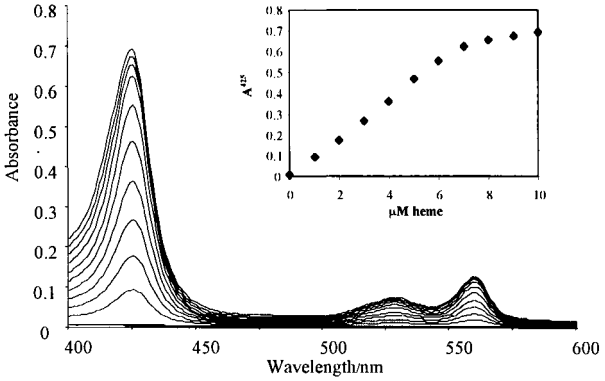


**Fig. 2.** Formation of reduced holo C11A/C14A cytochrome $c_{552}$ from apo protein (9 $\mu$M) and heme, added in increments of 1 $\mu$M, as monitored by visible absorption spectroscopy immediately after mixing of the samples. No change was observed in the spectra after longer incubation. (*Inset*) The change in $A_{425}$ with increasing amounts of heme.

of the apo C11A/C14A protein obtained from this method gave a weak spectrum, consistent with the presence of randomly coiled protein; there was no evidence of any $\alpha$-helical structure. These data suggested either that the heme extraction process irreversibly denatured the apo C11A/C14A or that the heme–protein interactions were necessary for the maintenance of the protein's tertiary structure. To distinguish between these possibilities, a less disruptive heme extraction procedure was sought.

Overnight incubation of concentrated C11A/C14A protein (0.3–0.4 mM) at 4°C with 1 M imidazole at pH 7.3 resulted in the formation of a red precipitate. After removal of the precipitate by centrifugation, followed by dialysis against 20 mM sodium phosphate (pH 7.3), 50% of the protein remained in solution; 1–2% of the total soluble protein was holo protein. The latter was removed by ion exchange chromatography as outlined in *Materials and Methods*. Apo C11A/C14A produced by this method was shown to reform the holo protein on addition of free heme, in both the oxidized and reduced states, within the mixing time (≈5 s) of the sample. Visible absorption spectra obtained from the reformed holo protein were indistinguishable from those of the initially isolated holo protein and showed no change over time. Titration of the apo protein with free heme under reducing conditions is shown in Fig. 2. The visible absorption spectra are identical with the reduced C11A/C14A protein as prepared, except that, at the end of the titration, some absorbance of free heme is apparent around 400 nm. The titration profile under oxidizing conditions was very similar to that shown (Fig. 2) for reducing conditions. The essentially stoichiometric incorporation of heme (Fig. 2) means that the dissociation constant for the hemoprotein must have an upper limit in the order of $10^{-9}$ M, comparable with values estimated for other *b* type cytochromes (14).

**Structure and Stability of C11A/C14A Protein in Holo and Apo Forms.** The gross secondary structures of the wild-type and C11A/C14A oxidized holo proteins seemed very similar as judged by the far UV CD spectra, whereas the apo form of the C11A/C14A mutant gave a different far UV CD spectrum (Fig. 3), indicative of a decrease in the $\alpha$-helical content. In a *b* type cytochrome, the heme can, in principle, take up one of two orientations (31) related by 180° rotation about the C5–C15 axis of the heme plane. The ratio of these orientations can be determined from the CD spectrum in the Soret region at 350–450 nm. The C11A/C14A protein gave a weakly positive signal, suggesting that one heme orientation was favored only slightly over the alternative. In wild-type cytochrome $c_{552}$, only one heme orientation occurs.

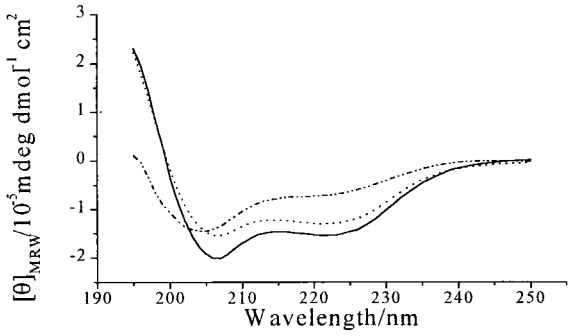


**Fig. 3.** Far UV CD spectra of oxidized holo wild-type cytochrome $c_{552}$ (solid line), oxidized holo C11A/C14A protein (dotted line), and apo C11A/C14A protein (dash-dot line). $[\theta]_{MRW}$, residue ellipticity.

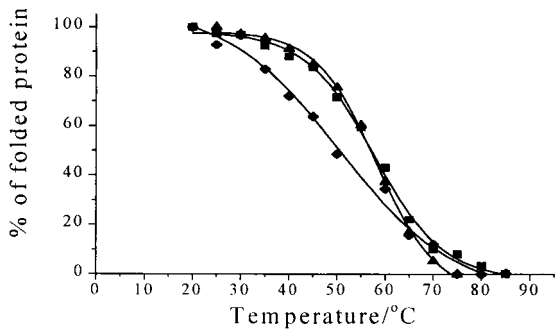


**Fig. 5.** Temperature-induced unfolding of oxidized holo and apo forms of C11A/C14A cytochrome $c_{552}$. Percentage of folded protein was calculated from the change in the CD signal at 222 nm for holo (■) and apo(♦) from the holo visible absorption at 416 nm (▲).

Differences in protein stability between C11A/C14A and the wild-type holo proteins were investigated by studying unfolding at a range of guanidine hydrochloride concentrations (Fig. 4) by monitoring changes in the visible absorption spectra. The unfolding of oxidized C11A/C14A holo protein was also followed by far UV CD spectroscopy and found to have the same unfolding profile as that observed by visible absorption spectroscopy (Fig. 5). All guanidine hydrochloride denaturations were reversible. It is clear that loss of the covalent attachment of heme results in a much less stable form, in both oxidized and reduced states, of the protein. The reduced form of the wild-type protein could not be denatured significantly by accessible concentrations of guanidine hydrochloride (Fig. 4).

Wild-type holo cytochrome $c_{552}$ is extremely stable to high temperature; the oxidized form is reported to withstand autoclaving and has a melting temperature ($T_m$) of 85.7°C in 1.5 M guanidine hydrochloride (13). The thermal stability of C11A/C14A protein was monitored by far UV CD spectroscopy and visible absorption spectroscopy for the holo protein or by far UV CD spectroscopy alone for the apo protein. The $T_m$ for the oxidized holo C11A/C14A protein in the absence of guanidine hydrochloride was found to be 58°C by both methods of determination; this temperature is only slightly higher than $T_m$ for the apo protein (51°C) as illustrated in Fig. 5. Direct comparison of the $T_m$ values with the value for the wild-type protein is not possible, because oxidized holo C11A/C14A protein is completely unfolded in 1.5 M guanidine hydrochloride, whereas wild-type protein is stable to temperatures in excess of 100°C in the absence of guanidine hydrochloride.

The relative stabilities of the wild-type and C11A/C14A cytochromes across a wide pH range were compared. The wild type was stable between pH 3 and pH 10, whereas the C11A/C14A protein was only stable between pH 5 and pH 8 (Fig. 6).

## Discussion

The substitution of two cysteines in the heme binding site of cytochrome $c_{552}$ from *H. thermophilus* by alanines has not caused a drastic structural change. We make this conclusion from the following. (*i*) The far UV CD spectra of the wild-type and the C11A/C14A protein are nearly identical. (*ii*) The reduction potential of the heme iron in the C11A/C14A protein is consistent with retention of histidine and methionine ligands. (*iii*) Reorganization of the mutated protein to permit other histidine and methionine ligands can be ruled out, because there are no alternative histidine residues in the sequence, and the only other methionine lies between the two alanines and is separated by two residues from the histidine. (*iv*) A preliminary NMR experiment on the reduced double alanine protein shows the characteristic resonance from a methyl group that is shifted out of the main spectral envelope by a ring current effect (T. Pertinhez and L. J. Smith, personal communication) and is typical of a methionine

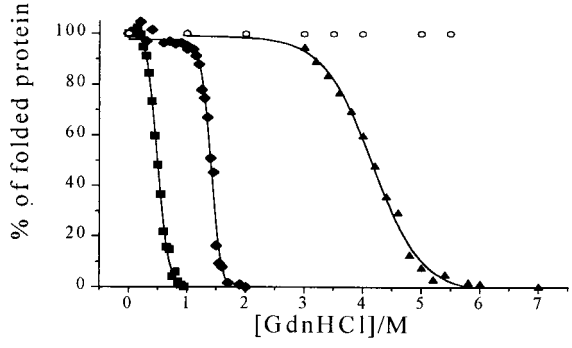


**Fig. 4.** Relative stability of oxidized wild-type protein (▲) and oxidized (■) and reduced (♦) forms of C11A/C14A protein to unfolding in guanidine hydrochloride. The percentage of folded protein was calculated for the oxidized and reduced forms from the changes in absorbance at 416 nm and 425 nm for oxidized and reduced C11A/C14A protein, respectively, and 410 nm for the oxidized wild-type protein. The reduced wild-type protein (○) was stable up to 5.5 M guanidine hydrochloride. A small amount of unfolding occurred from 6–7 M guanidine hydrochloride, but because the protein does not fully unfold, a meaningful percentage of folded protein cannot be calculated.

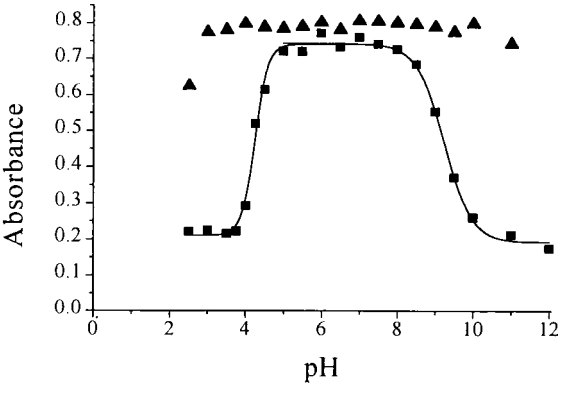


**Fig. 6.** pH-induced denaturation of 15 $\mu$M oxidized wild-type (▲) and C11A/C14A (■) cytochrome monitored by absorbance at 410 nm and 416 nm, respectively. Separate curves were fitted to the C11A/C14A data for pH 2–7.5 and pH 5–12.

ligated to a heme iron. Thus, there is every indication that the double alanine mutant has essentially the same structure as the parent cytochrome $c_{552}$.

This conclusion allows us to assess some of the consequences of the loss of the two covalent bonds between cysteine and heme. First, the effect on reduction potential is not dramatic. The shift to a less positive value suggests that the electron withdrawing groups of the vinyl groups in a *b* type are not dominant in setting the potential, otherwise the C11A/C14A protein should have had a more positive potential (1). However, we cannot exclude the effects of other factors, counteracting the presence of the vinyl groups, that result from the loss of the covalent bonds. The present work shows that the loss of covalent attachment leaves a protein from which heme does not readily dissociate. Thus, the idea that heme retention (30) is the main purpose of the major biosynthetic effort that is required to make a *c* type cytochrome does not receive support from the present work. Other reasons for the formation of *c* type cytochromes have been discussed recently (8). What is dramatically increased by the presence of the two thioether bonds in the cytochrome $c_{552}$ is the stability of the protein toward both thermal and chemical denaturation. In the absence of a high resolution structure of the C11A/C14A protein, it is not possible to say how much of the destabilization is due to the thioether bond loss and how much is due to a general loosening of the protein structure. However, it is clear that acquisition of the typical class I cytochrome *c* fold does not necessarily depend on the formation of thioether bonds. In the case of mitochondrial cytochrome *c*, the stabilizing effect of these bonds can be postulated to be crucial for adoption of the cytochrome *c* fold because of generally weaker packing interactions throughout the molecule.

The fact that the addition of heme to the apo C11A/C14A protein results in formation of a holo protein suggests that the apo form has either a heme pocket or elements of structure that recognize heme and allow folding to give the double alanine holo protein. The resulting protein seems to be a 1:1 mixture of isomers with heme inserted in either of the two possible orientations, which are related to one another by 180°. These observations have important consequences for the assembly of cytochrome $c_{552}$ in the *E. coli* cytoplasm. They imply that the apo wild-type protein would also take up a folded structure after noncovalent binding of heme. Such heme binding could then be followed by noncatalyzed formation of the two thioether bonds as suggested by Sambongi and Ferguson (9). However, the cytochrome $c_{552}$ formation in the cytoplasm results in a single product as judged by the CD spectrum in the Soret region and by NMR studies (P. D. Barker, personal communication), whereas the cytochrome *b* derivative contains both orientations. This difference is rationalized by assuming that only one noncovalent mode of interaction can lead to successful covalent bond formation, and thus by equilibrium dissociation and reassociation—or at least reorientation—of heme *in vivo*, a specific *c* type cytochrome is formed. Such a proposal would be strengthened if it could be shown that cytochrome $c_{552}$ can assemble correctly *in vitro* from apo protein and heme alone. Strictly speaking, until this experiment gives a positive result, it remains possible that the exceptional cytoplasmic formation of cytochrome $c_{552}$ in *E. coli* and of a second thermophilic cytochrome *c* (11, 12) depends fortuitously on the assistance of another cytoplasmic factor. If spontaneous heme attachment can follow its binding with no assistance from supplementary factors, then possibly the mitochondrial heme lyases act, at least in part, as specific chaperones and thus promote the adoption of a suitable conformation of the mitochondrial apo cytochrome *c*, which is ready to bind heme. It is more difficult to envisage such a role for the bacterial *c* type cytochrome biogenesis apparatus as the latter acts on a wide range of proteins (6, 7).

Cytochrome $c_{552}$ from *Thermus thermophilus* is a second thermostable *c* type cytochrome that can be expressed in the cytoplasm of *E. coli*. In contrast to *H. thermophilus* $c_{552}$, the *E. coli* expression of the *T. thermophilus* protein yields a heterogeneous mixture of holo cytochrome species. In one study, a species with heme attached through only one cysteine was obtained (12). In a second study, a mixture of holo cytochromes was obtained, including both a cytochrome $c_{552}$ species with both cysteines attached to the heme, as well as species with undefined chemical modifications to the heme moiety (11). Despite these complexities, experiments with *T. thermophilus* $c_{552}$ are consistent with the adoption of a prefolded state of the thermophilic apo protein that can bind heme before thioether bond formation.

Finally, it should be noted that in *H. thermophilus* itself cytochrome $c_{552}$, located in the periplasm, probably does not self-assemble. At the high growth temperatures, self-assembly would not be possible, because, as we have shown, the apo protein, as a C11A/C14A variant, is not stable above 42°C. The structure of this apo protein will be of great interest in the general context of research into cytochromes *c*.

We thank Y. Sambongi for providing pKHC12 and introducing us to cytochrome $c_{552}$, A. Willis for protein sequencing, and M. L. D. Page, J. W. A. Allen, and P. D. Barker for helpful discussions. This work was supported by the Biotechnology and Biological Sciences Research Council Grant C08007.

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