NMR techniques and 8-anilino-1-naphthalenesulphonate (ANS) binding studies have been used to characterize the apo state of a variant of cytochrome c₅₅₂ from Hydrogenobacter thermophilus. In this variant the two cysteines that form covalent thioether linkages to the heme group have been replaced by alanine residues (C11A/C14A). CD studies show that the apo state contains ~14% helical secondary structure, and measurements of hydrodynamic radii using pulse field gradient NMR methods show that it is compact (Rₓ, 16.6 Å). The apo state binds 1 mol of ANS/mol of protein, and a linear reduction in fluorescence enhancement is observed on adding aliquots of hemin to a solution of apo C11A/C14A cytochrome c₅₅₂ with ANS bound. These results suggest that the bound ANS is located in the heme binding pocket, which would therefore be at least partially formed in the apo state. Consistent with these characteristics, the formation of the holo state of the variant cytochrome c₅₅₂ from the apo state on the addition of heme has been demonstrated using NMR techniques.

The properties of the apo state of C11A/C14A cytochrome c₅₅₂ reported here contrast strongly with those of mitochondrial cytochrome c whose apo state resembles a random coil under similar conditions.

There has been much interest in characterizing the structural and dynamical properties of the apo forms of heme-binding proteins to gain insight into the stability, folding, and assembly of these ubiquitous proteins (e.g. see Refs. 1–3). Such studies are of particular significance for the c-type class of cytochromes where the heme group must become covalently bound to the protein to generate the functional native structure (4). For mitochondrial cytochrome c, the most studied of this class of proteins, both the local and global properties of the apo state in aqueous solution have been shown to resemble those expected for an unfolded random coil (5, 6), although more compact conformations with some helical secondary structure are observed at high salt concentrations (7) or in the presence of lipid membranes (8, 9). Furthermore, noncovalent binding of heme gives rise to a partially folded state, the heme group presumably acting as a site for nucleation of protein folding (10). Despite these findings heme has not been found spontaneously in vitro to attach covalently to the apo form of mitochondrial cytochrome c. It is, therefore, not possible to reconstitute the native holo structure simply by adding heme to the apo state of the protein.

Biophysical studies of the folding of mitochondrial cytochrome c have all been performed with intact thioether bonds between the two cysteines of the CXXCH motif and the heme group (11–13). It is not clear, however, to what extent the data from these studies relate to the situation in vivo where the covalent attachment of the heme to the protein has to take place at some stage during the folding process. In bacteria the covalent attachment of heme occurs physiologically in the periplasm where the cytochrome c biogenesis apparatus, which varies in complexity depending on cell type, is located (14, 15). An exception to this periplasmic location for the covalent heme attachment has been found for Hydrogenobacter thermophilus cytochrome c₅₅₂, which has the same fold (sometimes called class I) as mitochondrial cytochrome c. For H. thermophilus cytochrome c₅₅₂, the holoprotein is formed in the cytoplasm of Escherichia coli (16–18). The attachment of heme therefore occurs independently of the biogenesis apparatus in the periplasm. It has been proposed that holo H. thermophilus cytochrome c₅₅₂ is generated spontaneously, implying that the apo state of this protein has sufficient structure to form a binding site for heme, the occupancy of which could be followed by covalent bond formation (17). These proposed characteristics of H. thermophilus cytochrome c₅₅₂ are, therefore, significantly different from those reported for mitochondrial cytochrome c.

Further insight into the spontaneous formation of holo H. thermophilus cytochrome c₅₅₂ has come from the finding that replacement of the two cysteine residues of the CXXCH motif by alanines (C11A/C14A) results in a protein that binds heme noncovalently, i.e. in the formation of a cytochrome b variant of the cytochrome c₅₅₂ (19). This protein is the only example of a conversion of a c-type (defined as heme attached by two thioether bonds) to a b-type (defined as heme bound noncovalently) cytochrome through mutation and raises questions about the acquisition of the cytochrome c fold since the cytochrome b variant appears to have essentially the same native
structure as the original cytochrome c552, at least as judged by CD spectroscopy (19). Removal of the noncovalently bound heme from the cytochrome b variant generates an apoprotein that has an altered structure compared with the holo state, although CD spectroscopy suggests it is ~14% helical (compared with 43% helical structure in the holo state). The conformational properties of this apoprotein are of considerable interest, not least because studies by both visible absorption and CD spectroscopy show that addition of heme results in the spontaneous regeneration of the cytochrome b form of the protein (19). These observations suggest that the apoprotein has sufficient structure to provide at least a nascent heme binding site and that following insertion of the heme into that site the cytochrome c fold is adopted. In this paper we describe the results of a study of the b-type variant (C11A/C14A) of cytochrome c552 by NMR spectroscopy and ANS fluorescence measurements to probe directly the characteristics of its apo state. The experiments show that the apo form of C11A/C14A cytochrome c552 is compact and partially folded and suggest that it contains a nascent heme binding site.

EXPERIMENTAL PROCEDURES

Protein Samples—Wild type and C11A/C14A cytochrome c552 expressed as holoproteins from E. coli were prepared as reported previously, and C11A/C14A cytochrome c552 was prepared by incubating on ice for 4 h a solution of purified holo C11A/C14A cytochrome c552 in 20 mM sodium phosphate buffer at pH 7.3 containing 1 mM imidazole. The sample was then centrifuged to remove the heme moiety, and the supernatant was dialyzed extensively against 200 mM sodium phosphate buffer at pH 7.3 containing 1M imidazole. The purified apoprotein was then lyophilized and stored at −20 °C. Reconstitution of holo C11A/C14A cytochrome c552 for the NMR studies was achieved by adding a 150-μL aliquot of a 5 mM solution of hemin in deuterated Me2SO to a 500-μL sample of 1.4 mM apoprotein (in D2O, 20 mM phosphate buffer). The sample was then incubated overnight. All NMR samples contained 1.4 mM protein in D2O at pH 7.4. 20 μL of 1% dioxan was added to the samples used for the diffusion experiments. For the NMR experiments on the holoprotein in its reduced Fe(II) state, reduction was achieved by using sodium dithionite, and the Fe(II) state. Reduction was achieved by using sodium dithionite, and the spectrum characteristic of the holo state was obtained, thus providing evidence for native-like heme binding and formation of the holoprotein during the experiment. Fig. 1b shows the upfield region of the NMR spectra where the changes caused by the addition of heme are particularly marked. No resonances are visible in the spectrum of the apo form, but after the addition of heme well resolved resonances of the side chain protons of Met-59 are clearly visible. Some line broadening of these resonances is, however, evident, and one additional weak resonance (at ∼1.06 ppm) is also seen in this region after reconstitution that is not observed in the holo state spectrum. The assignment of this signal is not clear, but it and the line broadening effects may be the result of the presence of species with slight variations in the heme binding.

Pulse field gradient NMR methods provide a method for determining the effective hydrodynamic radii of native and non-native states of proteins (20, 21, 24). The rates of decay of the intensities of protein resonances relative to those of a small reference molecule (here dioxan) are measured as a function of increasing gradient strength. From the ratio of these decay rates (dreference/dprotein), the effective hydrodynamic radius of the protein can be calculated (21). Measurements of this type have been made for the holo form of wild type cytochrome c552 (in both its reduced Fe(II) and oxidized Fe(III) states) and for the C11A/C14A variant in its holo (reduced and oxidized) and apo forms and when denatured in 8 M urea (Table I).

The hydrodynamic radii of the holo states of wild type and C11A/C14A cytochrome c552 are very similar (14.3 ± 0.25 and 13.9 ± 0.25 Å, respectively, for the reduced state). For both proteins an increase in the hydrodynamic radius of ∼10% is observed for the oxidized compared with the reduced state of the holoprotein. This increase is comparable to the reported 8% increase in hydrodynamic radius on oxidation for mitochondrial cytochrome c measured by x-ray scattering (25). For cytochrome c the differences in the effective radii of the oxidized and reduced states are thought to result from a combination of electrostatic and conformational changes. Small structural differences have been identified between the two redox forms of cytochrome c (25, 26). In addition it has been suggested that phosphate binding to lysine-rich sites at low ionic strengths in the oxidized state may contribute to the increase in the effective

1 The abbreviation used is: ANS, 8-anilino-1-naphthalenesulphonate.

2 P. D. Barker, E. J. Tomlinson, N. Sinha, Y. Sambongi, and S. J. Ferguson, unpublished.
tive hydrodynamic radius (27). Similar effects are likely to be responsible for the differences in size of the oxidized and reduced states of cytochrome c reported here. Similarly the small differences observed between the hydrodynamic radii of the wild type and C11A/C14A variant of cytochrome c may arise from a combination of small structural variations and of differences in the hydration spheres of the wild type and mutant proteins.

Measurements for the apo form of C11A/C14A cytochrome c show that this form of the protein has an effective hydrodynamic radius of 16.6 ± 0.28 Å, an increase of less than 10% over the hydrodynamic radius of the oxidized form of the holo state. A significant expansion in the hydrodynamic radius of C11A/C14A cytochrome c to 25.5 ± 1.19 Å is, however, observed for the protein denatured in 8 M urea (a 67% increase in hydrodynamic radius compared with that of the oxidized holoprotein). The hydrodynamic radius of the latter is similar to that predicted for a fully denatured polypeptide chain of 80 amino acids (26.9 Å) by using the empirical relationship between the effective hydrodynamic radius and polypeptide chain length established from the analysis of data for a series of peptides and proteins under strongly denaturing conditions (21). The significantly smaller hydrodynamic radius for apo C11A/C14A cytochrome c compared with the urea-denatured state reported here shows that the global properties of the apo form are very different from those of an unstructured or random coil protein. The apo state will, like most non-native states, be an ensemble of interconverting conformers. The value of 16.6 ± 0.28 Å measured for the effective hydrodynamic radius of the apo state requires, however, that the majority of conformers within this ensemble of structures are highly compact.

The binding of the hydrophobic dye ANS to the holo, apo, and urea-denatured states of C11A/C14A cytochrome c has been probed using fluorescence emission spectroscopy. The emission of ANS is significantly increased when the molecule is bound in a nonpolar environment. ANS is therefore often used as a probe for exposed hydrophobic groups in proteins (22, 28, 29). For cytochrome c no fluorescence enhancement was observed for samples of the holo C11A/C14A protein or the urea-denatured protein, a result in accord with the fully folded and fully unfolded nature of these states. A significant enhancement of fluorescence intensity and a blue shift of the emission maximum was, however, observed for ANS in the presence of apo C11A/C14A cytochrome c (Fig. 2a).

A Compact Partially Folded Apo State of Cytochrome c

Fig. 1. a, one-dimensional 1H NMR spectra of the apo (upper panel) and holo (lower panel) forms of C11A/C14A cytochrome c measured using pulse field gradient NMR methods. b, the upfield region of the NMR spectra of the apo (upper panel) and holo (lower panel) forms of C11A/C14A cytochrome c and the product formed from adding hemin to the apo state of the protein to reconstitute the holo form (middle panel). The resonances from the side chain protons of Met-59 are labeled in the holo state spectrum. The NMR samples contained 1.4 mM protein in D2O at pH 7.4, and for the holo form the protein was in the reduced state.

|                  | \( \delta_{\text{reference}}/\delta_{\text{protein}} \) | \( R_h (\text{Å}) \) |
|------------------|-------------------|-------------------|
| Wild type protein holo reduced state | 6.75 ± 0.12 | 14.3 ± 0.25 |
| C11A/C14A variant holo reduced state | 6.57 ± 0.12 | 12.9 ± 0.25 |
| Wild type protein holo oxidized state | 7.55 ± 0.13 | 16.0 ± 0.28 |
| C11A/C14A variant holo oxidized state | 7.19 ± 0.12 | 15.3 ± 0.25 |
| C11A/C14A variant apo form | 7.83 ± 0.13 | 16.6 ± 0.28 |
| C11A/C14A variant in 8 M urea | 12.03 ± 0.56 | 25.5 ± 1.19 |

\(*\) The errors are estimated from repeat measurements as reported by Wilkins et al. (21).
binding sites per protein molecule is 1.01 ± 0.02 by analyzing the fluorescence intensity as a function of the concentration of added ANS. We then followed the changes in fluorescence emission of ANS in a sample of apo C11A/C14A cytochrome c₅₅₂ on the addition of hemin and observed a stepwise linear reduction in the fluorescence enhancement to a value indicative of the absence of specific binding of ANS (Fig. 2, b and c). This latter result suggests that the single ANS binding site is in the heme pocket of the apo state and that ANS is displaced by a heme group to form the holo state during the titration. The binding of ANS does not appear to alter significantly the structure of apo C11A/C14A cytochrome c₅₅₂ as no change in the far UV CD spectrum was observed for this state on the addition of ANS. Taken together, these observations suggest that ANS binds in effect exclusively in the heme binding pocket and therefore indicate that the heme pocket is at least partially formed in the apo state of the protein.

**DISCUSSION**

Non-native states of proteins are, in general, best described as ensembles of interconverting conformers. The reconstitution of the holo form of C11A/C14A cytochrome c₅₅₂ observed to take place following the addition of heme to a solution of the apoprotein could be anticipated if most of the conformers present in the apo state ensemble are partially folded and contain a nascent heme binding site. Alternatively, similar reconstitution could occur if unfolded random coil conformations predominated in the apoprotein ensemble, but these conformers were in rapid equilibrium with a small proportion of significantly folded protein. In this case heme binding by the folded structures would displace the conformational equilibrium in favor of further adoption of the folded state resulting in reconstitution of the entire population. The effective hydrodynamic radius of 16.6 ± 0.28 Å for the apo state of C11A/C14A cytochrome c₅₅₂ reported here provides strong evidence for the first of these proposals and that the population of highly unfolded states is very low. To obtain a measured value of the hydrodynamic radius within −10% of that of the native protein, the conformational ensemble of the apo state must contain predominantly highly compact conformers. The CD data show that these conformers contain, on average, −14% helical structure (compared with 43% in the native protein). The stoichiometric binding of ANS to the apoprotein and its reversal by the addition of hemin suggest that, in common with apohemoglobin and apomyoglobin, the ANS occupies at least part of a nascent heme binding site. However, strictly speaking the ANS binding site of the apo b-type variant of cytochrome c₅₅₂ could be separate from the heme binding site, with occupancy of the latter by heme causing abolition of the ANS binding site. This, however, seems the less likely of the two explanations for the reversal of ANS binding by heme.

These results for cytochrome c₅₅₂ contrast strongly with the conclusions of studies of mitochondrial cytochrome c that show that the apo state of this protein resembles a random coil under similar conditions. It is interesting to consider the reasons for the different characteristics of the apo states of these two proteins. Cytochrome c₅₅₂ comes from the thermophilic bacterium H. thermophilus that grows optimally at 70 °C, and the holo structure of wild type cytochrome c₅₅₂ is stable to temperatures in excess of 100 °C. The stability of mitochondrial cytochrome c is, however, lower with a denaturation temperature (Tᵥ) of 67 °C for the oxidized state (31). Comparisons have been made of the structures and sequences of homologous proteins from thermophiles and mesophiles (32) including a comparative study of cytochrome c₅₅₂ from H. thermophilus and the homologous cytochrome c₅₅₁ from mesophilic Pseudomonas aeruginosa (33, 34). One feature identified that may be important in promoting the thermal stability of H. thermophilus cytochrome c₅₅₂ is the high density of hydrophobic side chains in the core of this protein (34). It is possible that this extensive network of hydrophobic side chains in cytochrome c₅₅₂ may also be responsible, at least in part, for the compact partially folded nature of its apo state. Collapsed hydrophobic cores are in general recognized to be a characteristics of compact non-native states of proteins (30, 35–37), and the network of side chains in cytochrome c₅₅₂ may promote the formation of such a hydrophobic core even in the apo state of the protein.

In contrast to mitochondrial cytochrome c, the apo states of b-type cytochromes that have been studied, such as those of cytochromes b₅₆₂ and b₆₇, adopt conformations that are highly native-like (2, 38–40). These apo states contain almost native levels of secondary structure and have essentially the same folds as the holoproteins, although they do have a degree of disorder in their tertiary interactions, and residues in their heme binding pocket are solvent-exposed. The apo state of C11A/C14A cytochrome c₅₅₂ has significantly less structure than the apo forms of b-type cytochromes that have been char-
acterized. Most notably the apo state of C11A/C14A cytochrome c_{552} has 29% less helical secondary structure than the holo state. However, NMR studies of a number of non-native states of proteins have identified that compact conformers within a non-native ensemble are likely to have at least some highly native-like characteristics (41–43). It is therefore interesting to postulate that the partially folded ensemble of the apo state of cytochrome c_{552} may contain a significant population of conformers with a native-like overall fold despite the reduced amount of persistent secondary structure. This characteristic would be consistent with the proposed nascent heme binding pocket within the apo state and would provide a mechanism by which such conformers can readily convert into the native state following the binding of heme.

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The Cytochrome c Fold Can Be Attained from a Compact Apo State by Occupancy of a Nascent Heme Binding Site
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