Role of Phenylalanine-82 in Yeast Iso-1-cytochrome *c* and Remote Conformational Changes Induced by a Serine Residue at This Position†

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ABSTRACT: A three-dimensional structural analysis of the reduced form of the Ser-82 mutant protein of yeast iso-1-cytochrome *c* has been completed to 2.8-Å resolution. Replacement of Phe-82 with a serine residue results in conformational changes both near and remote from the mutation site. Those groups undergoing positional shifts near Ser-82 include Arg-13, Gly-83 and -84, and the CBB methyl of the heme group. Remote shifts are centered about the propionate of pyrrole ring A and principally involve Asn-52, Trp-59, and an internally buried water molecule, WAT-166. Placement of a serine side chain at position 82 also leads to the formation of a large solvent channel which substantially increases the solvent accessibility of the heme group. This would appear to account for the much lower reduction potential observed for this protein. The detrimental effect of Ser-82 on both the steady-state activity and the rate of electron transfer in complexation with cytochrome *c* peroxidase can also be interpreted in terms of the modified character of the region about the mutation site. The remote conformational changes observed appear to represent the equivalent of the initial conformational changes occurring as yeast iso-1-cytochrome *c* is converted to the fully oxidized state during an electron-transfer event. These results agree well with the proposal [Moore, G. R. (1983) *FEBS Lett. 161*, 171–175] that the trigger for conformational changes between oxidation states resides in the nature of the interactions between the heme iron atom and the pyrrole ring A propionate group. Overall, our results support suggestions that Phe-82 in the wild-type protein has at least three roles. These include the following: limiting solvent accessibility to the heme, thereby regulating the reduction potential of this protein dielectrically; facilitating the rate of electron transfer by providing the optimal medium along the transfer route; forming contact face interactions with redox partners to assist in the formation of the productive electron-transfer complex.

Although the mechanism of electron transfer mediated by cytochrome *c* has been intensively studied, it remains an unresolved biochemical problem of fundamental importance [see reviews by Mathews (1985) and Poulos and Finzel (1984)]. In yeast (*Saccharomyces cerevisiae*), two isozymes of cytochrome *c* occur. The most abundant form is yeast iso-1-cytochrome *c*, and the three-dimensional atomic structure of this protein has been recently elucidated in our laboratory (Louie et al., 1988). The tertiary structure of yeast iso-1-cytochrome *c* is highly homologous with those of other eukaryotic cytochromes *c* (Takano & Dickerson, 1981a; Ochi et al., 1983). However, the yeast protein does have a number of unique structural features. These include alternative conformations for residues −5 to +1 at the N-terminal end of the polypeptide chain, residues 19–26 which form a surface β loop on the His-18 ligand side of the central heme group, and residues 49–56 which are at the C-terminal end of a helical segment forming a part of the heme pocket.[1]

An extensive background of functional and genetic studies related to the expression and electron-transfer properties of yeast iso-1-cytochrome *c* is available. This arises from the versatility of the yeast system which offers several advantages over others in the study of electron-transfer reactions. For example, in contrast to other eukaryotic cytochromes *c*, many in vivo generated mutants of yeast iso-1-cytochrome *c* can be isolated and functionally characterized (Hampsey et al., 1986). In addition, yeast is amenable to site-directed mutagenesis techniques (Zoller & Smith, 1983; Smith, 1986), and it is possible to specifically design iso-1-cytochrome *c* mutants to probe the electron-transfer mechanism of this protein.

Thus far, a detailed definition of the mechanism of electron transfer carried out by eukaryotic cytochromes *c* has been hampered by either a lack of structural information for a given protein or an inability to systematically generate mutants in proteins for which structural data are available. To a certain extent, this deficit in corresponding structural and functional data on the same protein has been overcome by using amino acid sequence comparisons between cytochromes *c*. However, the interpretation of functional results based on such comparisons has proven difficult given the multiplicity of differences generally found between the primary sequences of cytochromes *c*.

With the elucidation of the three-dimensional atomic structure of the iso-1-cytochrome *c* from yeast, and the derivation of methods to construct specific mutants of this protein, for the first time a comprehensive system is in place to investigate the electron-transfer function of cytochrome *c*. The present study has examined the role of the invariant residue Phe-82 by determining the three-dimensional structure of the Ser-82 variant of yeast iso-1-cytochrome *c*. Comparison

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[1] The nomenclature used to describe the atoms of amino acid residues and those of the protoporphyrin IX heme prosthetic group of yeast iso-1-cytochrome *c* follows that of the Protein Data Bank (Bernstein et al., 1977). Numbering of amino acids along the polypeptide chain is based on a sequence alignment with tuna and rice cytochromes *c* (Louie et al., 1988).

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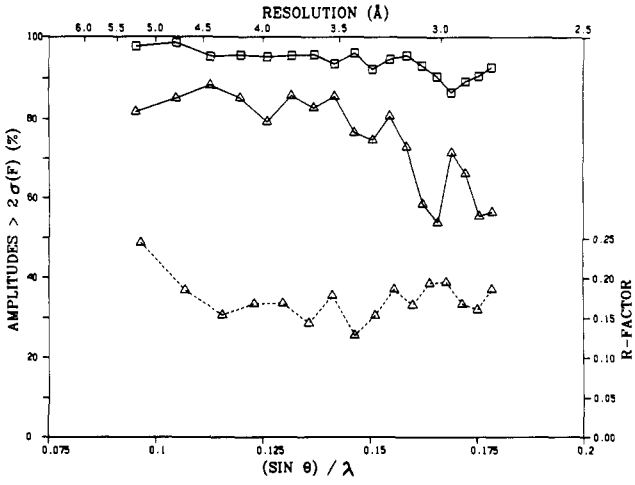

FIGURE 1: Plot of the percentage of structure factors greater than 2 times their estimated standard deviations over the 5.5–2.8-Å resolution range used in the present determination of the Ser-82 variant protein structure (Δ—Δ). Of the reflections measured, ~75% exceeded this limit and were considered observed. For comparison, a similar plot is presented for data from the much larger crystal used in the determination of the wild-type protein structure to 2.0-Å resolution (□). A further plot of the value of the conventional crystallographic *R* factor shows it to be fairly constant over the entire resolution range sampled (Δ---Δ). The overall *R* factor value obtained at the end of Ser-82 structure refinement was 0.17.

of the structural and functional attributes of the wild-type and mutant proteins has lead to the definition of several roles for Phe-82 in cytochrome *c*. The most important of these include preservation of the structural integrity of the heme pocket as well as involvement in the facilitation of electron transfer itself.

Materials and Methods

The Ser-82 variant protein of *Saccharomyces cerevisiae* iso-1-cytochrome *c* was prepared as previously described (Pielak et al., 1985, 1986). Diffraction-quality crystals of the reduced form of this protein were grown by using the free interface diffusion method (Salemme, 1972) employing conditions similar to those used to crystallize the wild-type protein (Sherwood & Brayer, 1985). Crystals formed in 1–2 days and attained a maximum size of 0.3 mm in each dimension over the period of a week. Precession photography and diffractometry on the Ser-82 variant crystals showed them to be isomorphous with crystals of wild-type iso-1-cytochrome *c*. Ser-82 variant crystals belong to the space group $P4_32_12$ with unit cell parameters of $a = b = 36.45$ (1) Å and $c = 136.48$ (3) Å. Crystals of the wild-type protein have unit cell dimensions of $a = b = 36.46$ (5) Å and $c = 136.86$ (12) Å.

Intensity data were measured on an Enraf–Nonuis CAD4-F11 diffractometer having a crystal to counter distance of 36.8 cm and a helium-purged path for the diffracted beam. The incident radiation was nickel filtered and generated from a copper target X-ray tube operated at 26 mA, 40 kV. Intensities were measured by using continuous ω scans 0.6° in width at a scan speed of 0.55° min^{-1}, with backgrounds taken as the terminal one-sixth of the total scan width on either side of each reflection. The ambient temperature during data collection was maintained at 15 °C. Diffraction data were corrected for backgrounds, absorption, crystal decay, and Lorentz and polarization effects by using methods previously described (Louie et al., 1988).

A complete 2.8-Å resolution diffraction data set was collected from a single crystal. This resolution limitation was the result of the relatively small size of Ser-82 variant crystals that could be obtained. Figure 1 shows the percentage of structure factors having amplitudes greater than 2 times their estimated standard deviations over the 5.5–2.8-Å range of data measured in the present structure determination. Of the 2198 structure factors in this range, 1645 (75%) had amplitudes exceeding the $\sigma = 2$ level and were considered observed. The effect of crystal size on the percentage of observed reflections is apparent in Figure 1, which shows a comparison with data obtained from the much larger crystal used to determine the 2.0-Å structure of the wild-type protein.

Table I: Stereochemistry of the Ser-82 Variant Yeast Iso-1-cytochrome *c* Structure

stereochemical refinement parameter	rms deviation from ideal values	refinement restraint weighting values
bond distances (Å)	0.014	0.020
angle distances (Å)	0.036	0.030
planar 1–4 distances (Å)	0.043	0.050
planes (Å)	0.014	0.020
chiral volumes (Å^3)	0.160	0.125
ω bond angles (deg)	2.8	3.0
single torsion contacts (Å)	0.216	0.250
multiple torsion contacts (Å)	0.217	0.250
hydrogen bond distances (Å)	0.231	0.250

The structure factor data set obtained for the Ser-82 variant protein was placed on an absolute scale by performing a linear rescale, based on F_{obsd}, against the same set of scaled 2.8-Å resolution structure factors used to elucidate the structure of wild-type iso-1-cytochrome *c*. A wild-type minus Ser-82 variant difference electron density map was calculated by using these scaled structure factor amplitudes. The wild-type iso-1-cytochrome *c* structure used to phase the difference map coefficients had previously been refined to 2.0-Å resolution with a final conventional crystallographic *R* factor of 0.20. The results of this latter structural analysis are to be published elsewhere. Since the computed difference electron density map indicated conformational rearrangements were localized to a few specific regions, a preliminary refinement model for the Ser-82 variant protein was constructed from the 2.0-Å wild-type structure described above. This was done by replacing the phenylalanyl side chain at position 82 in the wild-type structure with a serine side chain having the same χ_1 torsional angle. Included in this model were a total of 62 water molecules found outside the mutation site region in the structural analysis of the wild-type protein.

Refinement of this initial Ser-82 variant structure was carried out by using a restrained parameter least-squares procedure (Hendrickson & Konnert, 1981). Structure factors in the resolution range 5.5–2.8 Å and which were greater than $\sigma = 2$ of their estimated standard deviations were included in the refinement process. The course of refinement was assisted by two rounds of manual interventions based on fragment deleted, $F_o - F_c$ and $2F_o - F_c$, difference electron density maps. The positions of 10 additional water molecules were also determined and incorporated into the refinement model. Following a total of 30 cycles of least-squares refinement, the overall conventional crystallographic *R* factor was reduced to 0.17. As Figure 1 shows, the value for the *R* factor was fairly constant over the entire resolution range sampled. The final stereochemical parameters for the refined Ser-82 variant yeast iso-1-cytochrome *c* structure are tabulated in Table I, along with the restraint weighting values used in the last cycle of refinement.

Results

The present study shows replacement of Phe-82 in yeast

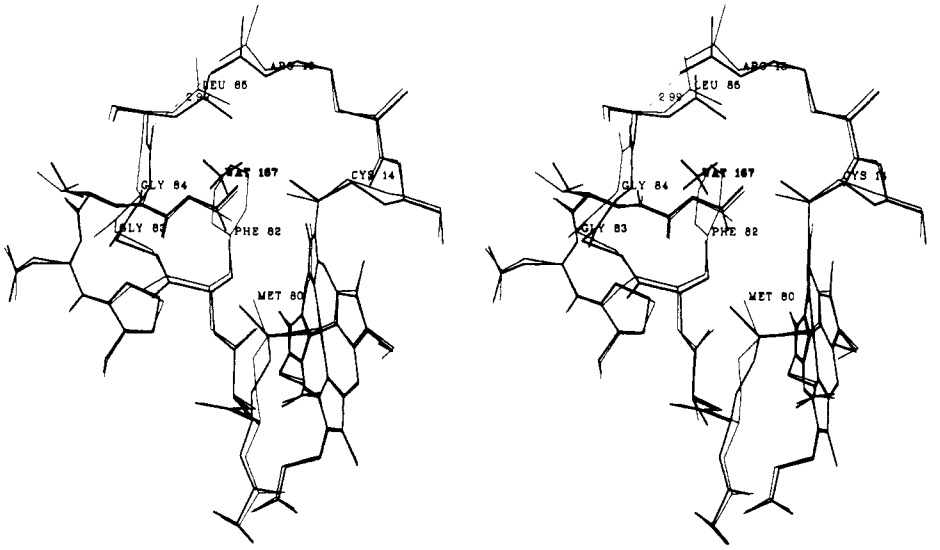

FIGURE 2: Composite stereo drawing showing the course of the polypeptide chain about residue 82 in the wild-type (thin lines) and Ser-82 mutant (thick lines) yeast iso-1-cytochrome *c* proteins. Also shown are the relative positions of the heme moieties of both molecules. Indicated by a dashed line and distance is the hydrogen bond formed between Arg-13 and the carbonyl group of Gly-84 in the Ser-82 variant protein. The position of the water molecule (WAT-167) found hydrogen bonded to Ser-82 in the mutation site has also been indicated.

iso-1-cytochrome *c* with a serine residue has an impact on the conformations of amino acids both near and remote from the mutation site. Since no definitive method is available for estimating the accuracy of a given set of protein atomic coordinates, a conservative approach is taken in discussing observed conformational changes. Only those shifts significantly greater than the average positional deviation of 0.26 Å found between all corresponding main-chain atoms of the wild-type and Ser-82 mutant proteins are noted.

As Figure 2 shows, the side chain of Phe-82 is packed in a coplanar fashion, next to the heme group in the wild-type protein (plane–plane separation is ~5 Å). Many of the amino acids in the immediate vicinity of Phe-82 move in the Ser-82 mutant protein. The most pronounced shifts involve Gly-83 and Gly-84 which pack closely against one side of the phenyl ring of this residue in the wild-type protein. These shifts are mediated in large part by alterations in the ψ angles of Ala-81 (Δ21°) and Gly-84 (Δ26°) and result in average positional displacements of 0.6 and 0.4 Å for Gly-83 and Gly-84, respectively.

A further reorientation involves the side chain of Arg-13. In the wild-type protein, the alkyl portion of the side chain of this residue packs against the extremity of the phenyl ring of Phe-82, and its guanidinium group is involved in a network of hydrogen bonds to other residues on the protein surface. As illustrated in Figure 2, the guanidinium group of Arg-13 reorients in the Ser-82 variant protein to form a hydrogen bond to the carbonyl group of Gly-84. This new interaction is made possible by two factors. First, the smaller side chain of Ser-82 permits Arg-13 to assume a conformation not allowed in the wild-type protein due to van der Waals contacts with the phenyl ring of Phe-82. Second, the inward movement of the polypeptide chain at residues 83 and 84 places these groups closer to Arg-13. Indeed, it is possible that the hydrogen-bond interaction formed by Arg-13 is a contributing factor in the shift of Gly-84. This interaction is also reflected in a significantly lower average thermal factor for the side chain of Arg-13 (18 Å^2) compared to that found in the wild-type protein (25 Å^2).

Overall, little difference is observed in the positions of heme atoms in the Ser-82 variant and wild-type yeast iso-1-cytochrome *c* structures. The average positional deviation for all heme atoms is 0.2 Å. An exception is the terminal CBB methyl group of pyrrole ring B which is adjacent to the thioether heme linkage to Cys-14. In the wild-type protein, this methyl group forms close contacts with the side chain of Phe-82 and is completely buried in the heme pocket. In the Ser-82 variant protein, this group becomes partially solvent accessible even though it shifts 0.8 Å further back into the hydrophobic heme pocket. As illustrated in figure 2, movement of the CBB heme methyl group is facilitated primarily by a readjustment in the geometry of the nearby thioether linkage to Cys-14.

Also perturbed in the Ser-82 variant protein are the terminal methyl groups of Met-80 (Δd = 0.7 Å) and Leu-85 (Δd = 0.7, 1.2 Å). In the wild-type protein, all three of these methyl groups are buried within the hydrophobic interior of yeast iso-1-cytochrome *c*, whereas in the Ser-82 variant protein they become solvent accessible. As with the CBB heme methyl group, the observed conformational changes in Met-80 and Leu-85 tend to minimize the degree of solvent exposure of these hydrophobic groups in the mutant protein.

A further set of conformational changes induced in the Ser-82 variant protein occur in an area far removed from the site of mutation. This region is centered about the carboxyl-terminal end of the heme propionate group of pyrrole ring A which is ~13 Å from the side chain of Ser-82. In the wild-type protein, the 01A oxygen atom of this propionate is hydrogen bonded to the hydroxyl group of Tyr-48 and two water molecules, which are in turn hydrogen bonded to Arg-38. The 02A oxygen atom forms hydrogen bonds to the backbone amide group of Gly-41 and the NE1 side chain nitrogen atom of Trp-59. As Figures 2 and 3 show, in the Ser-82 variant protein this propionate group retains a similar positioning and a comparable constellation of hydrogen-bonded interactions. One exception is the loss of the hydrogen bond to Trp-59 as the result of an ~15° rotation about the χ_1 torsional angle of this residue. This side chain movement causes the distance between the NE1 nitrogen atom of Trp-59 and the 02A oxygen atom of the heme propionate to lengthen from 3.0 Å in the wild-type protein to 3.7 Å in the Ser-82 variant protein. The overall average positional shift of atoms in the side chain of Trp-59 is 0.8 Å, with shifts greater than 1 Å occurring in the positions of the CZ2, CZ3, and CH2 carbon atoms. The

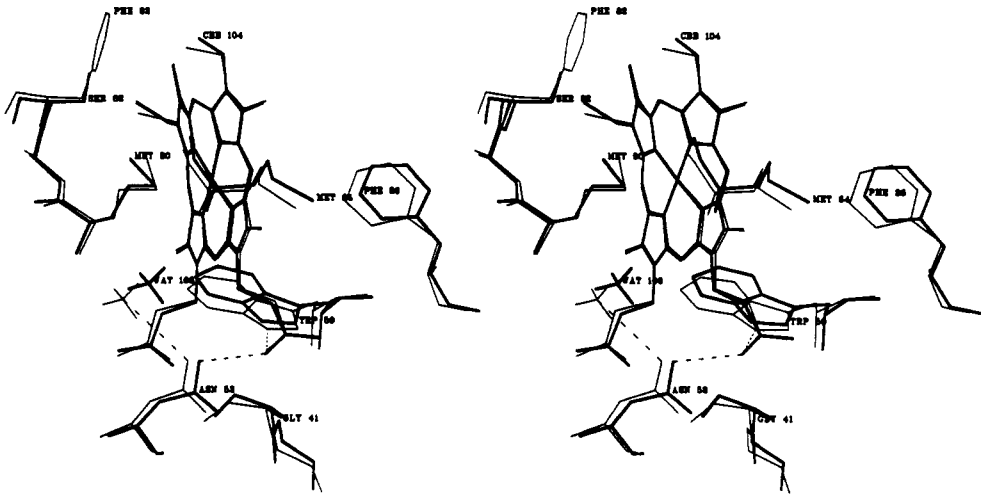

FIGURE 3: Composite stereo drawing of the structures of the wild-type (thin lines) and Ser-82 variant (thick lines) yeast iso-1-cytochrome *c* proteins in the region of the propionate group of pyrrole ring A. The exchange of a phenylalanine for a serine residue at sequence position 82 leads to two sets of concerted movements about this propionate. Only those hydrogen bonds affected by these movements are indicated by dashed lines. Since Ser-82 is far removed from this region, the observed conformational changes appear to occur in response to changes in the heme environment resulting from this mutation.

significance of this conformational change can be seen when compared with the relatively small overall average shift of 0.2 Å observed for the side chain atoms of the five other aromatic residues positioned in the heme pocket. Movement in the side chain of Trp-59 is further associated with concerted movements of two other side chains. As Figure 3 shows, one of these is the side chain of Met-64 which packs close to the side chain of Trp-59. The other is that of Phe-36 which is in turn packed adjacent to the side chain of Met-64. The majority of the displacement in the side chain of Met-64 involves the SD sulfur (Δd = 0.6 Å) and CE carbon (Δd = 0.8 Å) atoms. Atoms in the phenyl ring of Phe-36 move fairly uniformly by an average of 0.5 Å.

On the other side of the plane of the propionate group of pyrrole ring A, a further series of concerted movements occur in the Ser-82 variant protein. As illustrated in Figure 3, these involve the side chain of Asn-52 and an internally bound water molecule, WAT-166. In wild-type yeast iso-1-cytochrome *c*, WAT-166 forms hydrogen bonds to the side chains of Asn-52, Tyr-67, and Thr-78. In the Ser-82 variant protein, WAT-166 moves 1.4 Å in a direction almost directly toward the heme iron atom, decreasing the distance between these groups from 6.4 to 5.2 Å. In concert with this positional shift, the hydrogen bond between WAT-166 and Asn-52 is broken, and the side chain of this latter residue moves closer to the 02A propionate oxygen atom of the heme group. The overall shift observed for the side chain atoms of Asn-52 is 0.5 Å. Note that despite the movement of WAT-166, the hydrogen bonds formed to Tyr-67 and Thr-78 are retained and neither of these latter residues significantly shifts position.

Discussion

Effect of Heme Solvent Accessibility on Reduction Potential. It has been proposed that the dielectric constant of the heme environment is a significant factor in determining the redox potential of cytochrome *c* (Kassner, 1972). In this regard, both the polarity of residues in the heme pocket and the extent of heme solvent accessibility are primary factors. For wild-type yeast iso-1-cytochrome *c*, the heme group is observed to be almost completely buried, with only a few atoms along one edge of the heme group exposed to solvent (Louie et al., 1988). The reduction potential of this protein is +270 mV [e.g., see Wallace (1984) and Cutler et al. (1986)].

Phe-82 is an integral part of the hydrophobic pocket in which the heme is embedded and plays a major role in limiting solvent accessibility to this group. As Figure 4 shows, placement of a serine side chain at this position leads to the formation of a solvent channel extending down into the heme pocket. This substantially increases the solvent accessibility of the heme as well as that of a number of other internal residues which form a part of the heme pocket. The entrance to this new solvent channel is between the hydroxyl group of Ser-82, glycines-83 and -84, the side chain of Leu-85, and the CBB methyl of the heme. Located at the bottom of this solvent channel is the side chain of Met-80.

In the Ser-82 variant protein, the polarity of the heme environment is further increased by the hydroxyl function of Ser-82 and, in particular, by the readjustment of the side chain of Arg-13 closer to the heme group. This charged amino acid is now separated from the heme only by solvent molecules. Within the solvent channel itself, only one well-ordered water molecule can be resolved. It is hydrogen bonded to the OG oxygen atom of Ser-82. The absence of additional ordered water molecules is consistent with the hydrophobic nature of the side chains lining the remainder of this solvent channel.

Formation of a large solvent cavity, coupled with the introduction of several nearby polar groups, would be expected to significantly alter the dielectric constant within the heme pocket of the Ser-82 mutant protein, leading to a corresponding decrease in reduction potential. This is indeed the case, with the observed value being ~50 mV lower than that of the wild-type protein (Pielak et al., 1985, 1987). These results clearly establish Phe-82 as a major factor in forming the heme environment in cytochrome *c*. Furthermore, they emphasize the importance of the link between heme environment and reduction potential in this class of electron-transfer proteins.

Role of Phe-82 in Electron Transfer. Phe-82 has also been proposed to play a direct role in the electron-transfer reaction between yeast cytochrome *c* and yeast cytochrome *c* peroxidase (Poulos & Kraut, 1980; Poulos & Finzel, 1984). However, more recent work using site-directed mutagenesis techniques has shown that while Phe-82 may be involved, it is not essential to the process of electron transfer (Pielak et al., 1985, 1986). For example, these data show that the Ser-82 variant protein retains ~70% of the observed wild-type protein steady-state activity as substrate for oxidation catalyzed by cytochrome

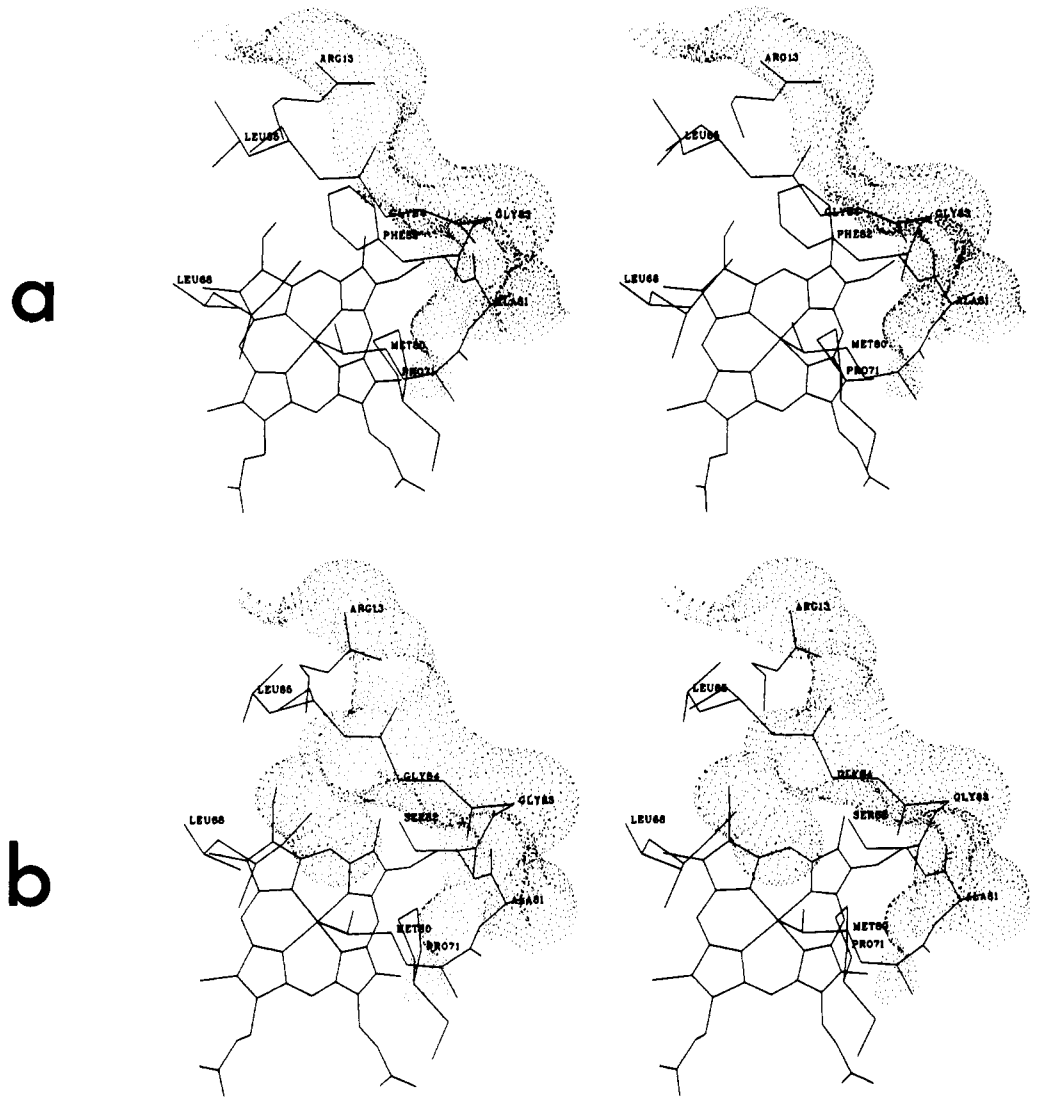

FIGURE 4: Stereo diagrams of the solvent-accessible surfaces of the (a) wild-type and (b) Ser-82 mutant yeast iso-1-cytochrome *c* proteins in the vicinity of the mutation site and the central heme moiety. Replacement of Phe-82 by a serine residue opens up a solvent channel which extends into the interior of the structure of yeast-iso-1-cytochrome *c*, significantly increasing the solvent accessibility of the heme group and of amino acids within the hydrophobic heme pocket. The direction of view shown is looking down the edge of the surface of both proteins and serves to illustrate the depth of the solvent channel present in the Ser-82 protein. The dot surfaces shown were calculated by using a probe sphere having a radius of 1.4 Å (Connolly, 1983).

c peroxidase. Nonetheless, these results do demonstrate that the precise character of the amino acid at sequence position 82 can have a substantial influence on electron transfer. This is apparent if one considers the simplest case where electron transfer is independent of the identity of this residue. In this situation, it would be reasonable to expect the activity of the steady-state reaction with cytochrome *c* peroxidase to actually increase with the Ser-82 variant protein since its lower reduction potential would facilitate the abstraction of an electron from the reduced mutant cytochrome *c* heme. Given that the reverse is observed, it is clear that the nature of residue 82 has an important modulating effect on some component of the reaction wherein oxidation by H_2O_2 is catalyzed by cytochrome *c* peroxidase. Further support for this arises from the observation that other amino acids substituted at this position exhibit a wide range of steady-state rates for the cytochrome *c* peroxidase catalyzed reaction and that these activities do not correlate with the reduction potentials measured for these mutant proteins (Pielak et al., 1985).

The role Phe-82 might play in electron transfer is suggested from structural studies of cytochrome *c* and the modeling of cytochrome *c*–cytochrome *c* peroxidase complexation. The coplanar placement of the phenyl ring of Phe-82 adjacent to the heme has led to the proposal that the delocalized π-electron systems of these groups are coupled, thereby providing a potential route for an electron to travel during its transfer to cytochrome *c* peroxidase (Poulos & Finzel, 1984). Modeling studies indicate Phe-82 is at the putative contact surface with cytochrome *c* peroxidase.

Further evidence of the role of Phe-82 can be found in studies of the rate of electron transfer occurring following complexation of reduced yeast iso-1-cytochrome *c* and the π-cation radical of cytochrome *c* peroxidase containing Zn-substituted heme (Liang et al., 1987). It has been shown that a phenylalanine or tyrosine residue at sequence position 82 results in an electron-transfer rate from ferrous cytochrome *c* which is 10^4 times greater than if a serine or glycine residue is present at this position. These results support the proposal that for the cytochrome *c*/Zn cytochrome *c* peroxidase reaction an aromatic side chain at this location facilitates the process of electron transfer through direct interaction with the heme π-electron system of cytochrome *c*.

In light of the available data, the detrimental effect of Ser-82 both on steady-state activity and on the rate of electron transfer

in the cytochrome c–Zn^{+0} cytochrome c peroxidase complex can be interpreted in terms of the modified character of this region of the protein. This mutant lacks the delocalized π-electron system of Phe-82 and would therefore be unable to facilitate electron transfer from the heme via this mechanism. Further, the smaller size of Ser-82 results in the formation of a solvent channel (Figure 4) at the former location of the phenyl ring of Phe-82. Thus, if an electron is to travel this route to cytochrome c peroxidase, it will encounter a substantially different medium in the Ser-82 mutant protein. Indeed, it is possible that disruption of the protein structure near position 82 in this mutant protein could result in electron transfer occurring via an entirely different mechanism (Marcus & Sutin, 1985).

Remote Conformational Changes. Moore (1983) has proposed the trigger for conformational changes between oxidation states in cytochrome c residues in differences in the interactions between the heme iron atom and the pyrrole ring A propionate group. These differences result in movements in the atomic positions of the groups interacting with this propionate, which are further transmitted to other parts of the protein involved in electron transfer. In this way, cytochrome c assumes the most suitable folding conformation compatible with its current oxidation state. Therefore, the conformations of residues in this network of movements would be expected to provide a good indication of the oxidation state of cytochrome c.

This proposal agrees well with the current structural studies of the reduced form of the Ser-82 variant protein. Replacement of Phe-82 has decreased the ability of this protein to stabilize the "reduced" conformation of the protein. This is apparent in the lower reduction potential measured. In this situation, the reduced form of the Ser-82 variant protein could be expected to assume a conformation tending toward that of the oxidized structure of the wild-type protein. A clear indication of this is reflected in the observed movement of the side chain of Trp-59, a residue known to be sensitive to the oxidation state of the heme (Takano & Dickerson, 1981a,b; Rackovsky & Goldstein, 1984; Wand & Englander, 1985). Thus, it seems likely that the shifts of Trp-59 and other amino acids in the bottom of the heme pocket in the Ser-82 variant protein represent the equivalent of the initial conformational changes occurring as yeast iso-1-cytochrome c is converted to the fully oxidized state during an electron-transfer event.

For the Ser-82 variant protein, it could be argued that increased heme solvent accessibility has affected the nature of the interaction between the heme iron atom and the propionate group of pyrrole ring A, thereby leading to a protein conformation approximating the oxidized state. The solvent channel adjacent to the heme group in this mutant protein, as well as the close proximity of Arg-13, might lead to a redistribution of the electrons within the delocalized π-electron system of the heme (Satterlee et al., 1987). This in turn would affect the ability of the heme to acquire an electron or transfer an electron to another redox partner. Heme solvent accessibility could also affect the character of the propionate group hydrogen bonded to Trp-59 in the wild-type protein. The breakage of this bond indicates the carboxyl function of this group has lost some of its negatively charged character and has become a poorer electron donor. Under these conditions, the interaction between the heme iron atom and this propionate group is likely to be different from that in the wild-type protein. As Moore (1983) has proposed, these variations in the electrostatic interactions of the heme could serve not only to lower the reduction potential of the heme but also to shift the structure of the Ser-82 variant protein toward that of the oxidized state of this protein. This proposal correlates well with the observed lower reduction potential of the Ser-82 mutant protein (Pielak et al., 1985) and the current observations of the conformational state of this protein.

A further set of movements in the structure of the reduced Ser-82 mutant protein are consistent with a shift toward a conformation expected for the oxidized state of the wild-type protein. These involve the side chain of Asn-52 and an internally buried water molecule (WAT-166) on the opposite side of the propionate group of pyrrole ring A from Trp-59. As Figure 2 illustrates, in the Ser-82 variant protein, WAT-166 moves by over 1.4 Å in a direction almost directly toward the heme iron atom. This positional shift leads to the breaking of the hydrogen-bond interaction between WAT-166 and Asn-52. A close analogue of WAT-166 is found in tuna cytochrome c (Takano & Dickerson, 1981a). A large shift in this internal water molecule is observed (1.0 Å) on going from the reduced to the oxidized state of the tuna protein. The direction of this movement parallels that found in the Ser-82 variant protein and occurs in response to the strong attraction of the positive charge resident on the heme iron atom in the oxidized state of tuna cytochrome c. This interaction is also believed to be one of several concerted movements which shift the overall conformation of cytochrome c toward stabilization of the oxidized state following electron transfer (Takano & Dickerson, 1981a,b; Rackovsky & Goldstein, 1984).

In summary, the current structural study and the results of other functional analyses suggest that Phe-82 plays at least three roles in yeast iso-1-cytochrome c. First, this residue limits the degree of solvent accessibility to the heme group and thereby assists in the regulation of the reduction potential of this protein dielectrically. Second, the aromatic side chain of this residue serves as an optimal medium along the electron-transfer route and greatly facilitates the rate of transfer of electrons to other redox proteins such as cytochrome c peroxidase. Third, this residue forms appropriate interactions with the contact face of redox partners and in this way assists in the formation of a productive electron-transfer complex.

The current study also serves to emphasize two further points concerning structure–function mutagenesis experiments. First, individual amino acids within protein structures can play both structural and functional roles, and these may be difficult to differentiate, particularly if other than single-site mutants are examined. Second, the present results demonstrate that the mutation of a single amino acid can induce conformational changes in regions remote from the mutation site. Both of these observations are consistent with the synthesis of proteins as specifically designed integral units, in which the placement and role of any given individual amino acid can be dependent on the conformation of other amino acids even if the latter are at some distance in the tertiary structure.

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Registry No. Phe, 63-91-2; Ser, 56-45-1; heme, 14875-96-8; cytochrome c, 9007-43-6.

REFERENCES

Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, E. F., Bruce, M. D., Rodgers, J. R., Kennard, O., Shimanouci, T., & Tasumi, M. (1977) *J. Mol. Biol. 112*, 535–542.

Connolly, M. L. (1983) *Science (Washington, D.C.) 221*, 709–713.

Cutler, R. L., Pielak, G. J., Mauk, A. G., & Smith, M. (1987) *Protein Eng. 1*, 95-99.
Hampsey, D. M., Das, G., & Sherman, F. (1986) *J. Biol. Chem. 261*, 3259-3271.
Hendrickson, W. A., & Konnert, J. (1981) in *Biomolecular Structure, Function, Conformation and Evolution* (Sunivasan, R., Ed.) Vol. 1, pp 43-57, Pergamon, Oxford.
Kassner, R. J. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2263-2267.
Liang, N., Pielak, G. J., Mauk, A. G., Smith, M., & Hoffman, B. M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1249-1252.
Louie, G. V., Hutcheon, W. L. B., & Brayer, G. D. (1988) *J. Mol. Biol. 199*, 295-314.
Marcus, R. A., & Sutin, N. (1985) *Biochim. Biophys. Acta 811*, 265-322.
Mathews, F. S. (1985) *Prog. Biophys. Mol. Biol. 45*, 1-56.
Moore, G. R. (1983) *FEBS Lett. 161*, 171-175.
Ochi, H., Hata, Y., Tanaka, N., Kakudo, M., Sakuri, T., Achara, S., & Morita, Y. (1983) *J. Mol. Biol. 166*, 407-418.
Pielak, G. J., Mauk, A. G., & Smith, M. (1985) *Nature (London) 313*, 152-153.
Pielak, G. J., Oikawa, K., Mauk, A. G., Smith, M., & Kay, C. M. (1986) *J. Am. Chem. Soc. 188*, 2724-2727.
Pielak, G. J., Concar, D. W., Moore, G. R., & Williams, R. J. P. (1987) *Protein Eng. 1*, 83-88.
Poulos, T. L., & Kraut, J. (1980) *J. Biol. Chem. 255*, 10322-10330.
Poulos, T. L., & Finzel, B. C. (1984) *Pept. Protein Rev. 4*, 115-171.
Rackovsky, S., & Goldstein, D. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5901-5905.
Salemme, F. R. (1972) *Arch. Biochem. Biophys. 151*, 533-539.
Satterlee, J. D., Moench, S. J., & Erman, J. E. (1987) *Biochim. Biophys. Acta 912*, 87-97.
Sherwood, C., & Brayer, G. D. (1985) *J. Mol. Biol. 185*, 209-210.
Smith, M. (1986) *Philos. Trans. R. Soc. London, A 317*, 295-304.
Takano, T., & Dickerson, R. E. (1981a) *J. Mol. Biol. 153*, 79-94.
Takano, T., & Dickerson, R. E. (1981b) *J. Mol. Biol. 153*, 95-115.
Wallace, C. J. A. (1984) *Biochem. J. 217*, 595-599.
Wand, A. J., & Englander, S. W. (1985) *Biochemistry 24*, 5290-5294.
Zoller, M. J., & Smith, M. (1983) *Methods Enzymol. 100*, 468-500.

Symmetry of the Inhibitory Unit of Human α_2-Macroglobulin†

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ABSTRACT: Human α_2-macroglobulin (α_2M) of M_r ~720000 is a proteinase inhibitor whose four identical subunits are arranged to form two adjacent inhibitory units. At present, the spatial arrangement of the two subunits which form one inhibitory unit (the functional "half-molecule") is not known. Treatment of α_2M with either 0.5 mM dithiothreitol (DTT) or 4 M urea results in dissociation of the native tetramer into two half-molecules of M_r ~360000. These half-molecules retain trypsin inhibitory activity, but in each case, the reaction results in reassociation of the half-molecules to produce tetramers of M_r ~720000. However, when reacted with plasmin, the preparations of half-molecules have different properties. DTT-induced half-molecules protect the activity of plasmin from inhibition by soybean trypsin inhibitor (STI) without reassociation, while urea-induced half-molecules show no ability to protect plasmin from reaction with STI. High-performance size-exclusion chromatography and sedimentation velocity ultracentrifugation studies were then used to estimate the Stokes radius (R_e) of α_2M and both DTT- and urea-induced half-molecules of α_2M. The R_e of tetrameric α_2M was 88-94 Å, while that of DTT-induced half-molecules was 57-60 Å and urea-induced half-molecules 75-77 Å. These results demonstrate that DTT- and urea-induced half-molecules have fundamentally different molecular dimensions as well as inhibitory properties. The hydrodynamic data suggest that the urea-induced half-molecule is a "rod"-like structure, although it is not possible to predict the three-dimensional structure of this molecule with the available data. The data are most consistent with the conclusion that the DTT-induced half-molecule is a "basket"-like structure, and this half-molecule is the basic inhibitory unit of α_2M.

The α-macroglobulins are a group of high molecular weight proteins that bind and inhibit a diverse variety of proteinases including members from all four catalytic classes [for reviews, see Roberts (1986) and Sottrup-Jensen (1987)]. Their inhibitory capacity results from a conformational change following proteinase reaction that appears to physically entrap the reacting proteinase molecule(s) (Barrett & Starkey, 1973), and they are therefore very different from the other, active-site-directed protein inhibitors of proteinases (Travis & Salvesen, 1983).

Human α_2-macroglobulin (α_2M) is the most intensively studied α-macroglobulin. α_2M is a tetrameric molecule formed by the noncovalent association of two disulfide-bonded subunits (Harpel, 1973). The primary structure of each M_r ~180000

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