Protein Structure, Folding, and Design 2, pages 167–173
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STRUCTURE-FUNCTION STUDIES ON YEAST HEME PROTEINS USING OLIGONUCLEOTIDE MUTAGENESIS

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ABSTRACT Oligonucleotide-directed mutagenesis has been applied to structure-function studies on two heme proteins of the yeast *Saccharomyces cerevisiae*, cytochrome *c* peroxidase and iso-1-cytochrome *c*. In the case of cytochrome *c* peroxidase, replacement of Trp-51 and Met-172 by Phe and Ser, respectively, has shown that neither residue is required for formation of the product of hydrogen peroxide oxidation, compound ES (although the physical properties are different in the mutants) and both mutant forms of the peroxidase are enzymatically active (the Phe-51 mutant has a major shift in pH optimum and a five-fold higher specific activity). In the case of iso-1-cytochrome *c*, Cys-107 has been replaced by Thr. This results in a form of cytochrome *c* that is more stable during redox reactions. The invariant residue, Phe-87, has been replaced by Tyr, Ser or Gly. All three variants are functional *in vivo* and *in vitro*. However, the non-aromatic substitutions cause a marked decrease in redox potential. Additional studies, in collaboration with the groups of B.M. Hoffman and C.M. Kay, have shown that removal of the aromatic residue at position 87 results in a decrease in the rate of electron transfer from Fe^{+2} iso-1-cytochrome *c* by four orders of magnitude and that, in the wild-type (Phe) protein, the polypeptide lying close to the heme has a unique conformation.

CYTOCHROME c PEROXIDASE

The chromosomally encoded gene for this mitochondrial protein has been isolated from *Saccharomyces cerevisiae* by molecular cloning (1). The chromosomal gene can be disrupted, resulting in a strain of yeast which does not produce the (nonessential) cytochrome *c* peroxidase and which is a suitable host for the expression of plasmid-borne mutant cytochrome *c* peroxidase genes (1,2). The enzyme is of particular interest because it is almost unique in that it is thought to have a protein-centred free radical species as an intermediate in its catalysis of the oxidation of Fe^{+2} cytochrome *c* by hydrogen peroxide (3). Its molecular structure has been established at atomic resolution (4,5) and spectroscopic studies have led to suggestions that the protein-centred free radical resides on a sulphur radical or on an aromatic side chain (6,7,8). Because of their proximity to the heme residue, Met-172 and Trp-51 have been postulated as the possible locations of the radical. The present study was undertaken to replace these residues to establish the effect of replacement on the electron paramagnetic resonance (EPR) signal which is characteristic of the radical and on the catalytic properties of the enzyme. A number of replacements have been constructed. Particularly of interest are Ser-172 and Phe-51; these residues occupy the homologous positions in the amino-acid sequence of horse radish peroxidase (9). The mutants where Met-172 is replaced (by Ser or Cys) were produced by oligonucleotide mutagenesis, using the two primer method (10), of a fragment of the cytochrome *c* peroxidase gene cloned into the plasmid-single strand vector pEMBL8(+) (11). The mutant fragments of the gene were transferred into the yeast expression vector YEp13CCP (12) and the gene expressed in yeast. The mutant cytochrome *c* peroxidases were isolated from the transformed yeast by standard procedures (2). Examination of the EPR signal of the oxidized form of the enzyme (compound ES) revealed that the Ser-172 mutant exhibited the signal (at 93°K) which is characteristic of the wild-type enzyme; however the signal is distinctly sharper (2). The catalytic properties of the wild-type enzyme and of the two mutants are identical within experimental error (2). Thus, replacement of Met-172 by Ser or Cys demonstrates that the Met residue is not critical to

peroxidase function. The effect of replacement of the Met residue on the EPR spectrum of compound ES will be discussed below.

Mutants where Trp-51 is replaced were produced by a new mutagenic strategy which takes advantage of the high efficiency of the dU-DNA method for oligonucleotide mutagenesis (13). The single-strand dU-DNA target for mutagenesis was derived from the pEMBL8(+) clone of the cytochrome _c_ peroxidase gene fragment (2) and it was mutated using oligonucleotides containing a mixture of nucleotides at the positions corresponding to the Trp codon (14). The Phe, Met, Thr, Cys and Lys replacements of Trp-51 were obtained in this way; screening by DNA sequence determination was possible because of the high efficiency of the dU-DNA procedure. All of the mutant cytochrome _c_ peroxidases, obtained by expression of the mutant gene in yeast, were enzymatically active. The catalytic properties of the Phe-51 mutant were examined in detail. Its pH optimum is shifted from that of the wild-type enzyme, pH 5.0, to pH 7.0 and its turnover rate at the optimum pH is increased five-fold (14). By comparison, the pH optimum and turnover-rate of the Ser-172 mutant do not differ significantly from those of the wild-type enzyme (14). The EPR spectrum of the Phe-51 compound ES at 93°K is like that of the Ser-172 mutant, lacking the shoulders seen at this temperature with the wild-type enzyme. However, at 10°K the spectra of the compounds ES derived from wild-type, Ser-172 and Phe51 cytochrome _c_ peroxidases are almost identical. Thus, the broad signal is not lost in the mutants; the physical properties of the mutant enzymes are changed so that the signal is not detected at 93°K (14). Because the two residues lie close to and on opposite sides of the heme residue, it appears that the radical's properties are under the influence of the heme.

In summary, replacement of Trp-51 and Met-172 in cytochrome _c_ peroxidase has demonstrated that the EPR signal in compound ES does not originate on these residues although they do influence its physical properties. Further, the Trp-51 residue has a very clear role in establishing the pH optimum and turnover rate of the enzyme.

CYTOCHROME c

Cytochrome _c_ is one of the most extensively studied of proteins. The sequences of over 90 different

mitochondrial cytochromes *c* have been established; there are twenty three totally conserved residues (15). While the functions of a number of these residues have been established by chemical modification, the role(s) of most of these residues can only be inferred from their location in the three-dimensional structure of the protein (16) and from the chemical function of their side chains. The properties of these residues can be studied by site-directed mutagenesis; an example of a residue that has been studied in this way is Phe-87 of yeast iso-1-cytochrome *c*. The gene for this cytochrome was isolated from a yeast genomic library using a synthetic oligonucleotide probe (17,18). The gene, carried in a yeast plasmid vector, can be expressed in a strain of yeast in which the two chromosomal loci, *CYC1* and *CYC7*, which encode iso-1-cytochrome *c* and iso-2-cytochrome *c*, are inactive (19). Mutants, with Phe-51 replaced by Tyr, Ser or Gly, have been constructed by two-primer oligonucleotide mutagenesis of the gene propagated in an M12mp vector (10,20). The mutant genes give products which support respiration in yeast in *vivo*, showing that the cytochromes *c* are at least partially functional in electron transfer. *In vitro* studies on the purified mutant cytochromes *c* have revealed important differences in properties. For example, the redox potentials of the wild-type and Tyr variants are very similar whereas the redox potentials of the Ser and Gly variants are about 50 mVs less (approximately +220 mV as contrasted with +270 mV) (20). This suggests that one of the roles of the Phe-87 residue is to establish a lipophillic shell around the heme which results in the higher redox potential. The Phe-87 residue lies on the face of the cytochrome *c* involved in electron transfer reactions. Thus, it is possible that it has a mechanistic role in transfer (5). This possibility has been investigated in a collaborative study with N. Liang with B.M. Hoffman (21). These studies have demonostrated that the wild-type and Tyr-87 variants of Fe^{+2}-iso-1-cytochrome *c* transfer an electron to $(Zn\text{-cytochrome } c \text{ peroxidase})^{+}$ four orders of magnitude more rapidly than do the Ser-87 and Gly-87 variants! Thus, the redox properties of wild-type iso-1-cytochrome *c* and the Tyr-87 variant as well as their properties in electron transfer are virtually identical. This raises the question of why Tyr has not appeared at this position in naturally-occurring mitochondrial cytochromes *c*. There is no direct answer to this

255, 8199-8205; Poulos, T.L. and Finzel, B.S. (1984). In: Peptide and Protein Reviews (M.T.W. Hearn, ed.).Marcel Decker, Inc., New York, N.Y., Vol. 4, pp. 115-171.

6. Hoffman, B.M., Roberts, J.E., Brown, T.G., Kang, C.H. and Margoliash, E. (1979). Electron-nuclear double resonance of the hydrogen peroxide compound of cytochrome c peoxidase: identification of the free radical site with a methionyl cluster. Proc. Natl. Acad. Sci. U.S.A. 76, 6132-6136.
7. Yonetani, T., Schleyer, H. and Ehrenberg, A. (1986). Studies on cytochrome c peroxidase VII. Electron paramagnetic resonance absorptions of the enzyme and complex ES in dissolved and crystalline forms. J. Biol. Chem. 241, 3240-3243.
8. Hori, H. and Yonetani, T. (1985). Powder and single-crystal electron paramagnetic resonance studies of yeast cytochrome c peroxidase and its peroxide compound ES. J. Biol. Chem. 260, 349-355.
9. Takio, K., Titani, K., Ericsson, L.H. and Yonetani, T. (1980). Primary structure of yeast cytochrome c peroxidase II. The complete aminoacid sequence. Arch. Biochem. Biophys. 203, 615-629.
10. Zoller, M.J. and Smith, M. (1984). Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. DNA 3, 479-488.
11. Dente, L., Cesareni, G. and Cortese, R. (1983). pEMBL: a new family of single strand plasmids. Nucleic Acids Res. 11, 1645-1655.
12. Broach, J., Strathern, J. and Hicks, J. (1979). Transformation in yeast, development of a hybrid cloning vector and isolation of the CAN1 gene. Gene 8, 121-133.
13. Kunkel, T.A. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. U.S.A. 82, 488-492.
14. Goodin, D.B., Mauk, A.G. and Smith, M. (1987). The peroxide complex of yeast cytochrome c peroxidase contains two distinct radical species, neither of which resides at methionine-172 or tryptophan-51. J. Biol. Chem. 262, in press.
15. Cutler, R.L., Pielak, G.J., Mauk, A.G. and Smith, M. (1987). Replacement of cysteine-107 of Saccharomyces cerevisiae iso-1-cytochrome c with threonine: improved stability of the mutant protein. Prot. Eng. 1, 95-99.

16. Tanako, T. and Dickerson, R.E. (1981). Conformation change of cytochrome c. II. Ferricytochrome c refinement at 1.8 A° and comparison with the ferrocytochrome c structure. J. Mol. Biol. 153, 95-115.
17. Gillam, S., Rottman, F., Jahnke, P. and Smith, M. (1977). Enzymatic synthesis of a segment of yeast iso-1-cytochrome c gene. Proc. Natl. Acad. Sci. U.S.A. 74, 96-100.
18. Montgomery, D.L., Hall, B.D., Gillam, S. and Smith, S. (1978). Identification and isolation of the yeast cytochrome c gene. Cell 14, 673-680.
19. Faye, G., Leung, D.W., Tatchell, K., Hall, B.D. and Smith, M. (1981). Deletion mapping of sequences essential for *in vivo* transcription of the iso-1-cytochrome c gene. Proc. Natl. Acad. Sci. U.S.A. 78, 2258-2262.
20. Pielak, G.J., Mauk, A.G. and Smith, M. (1985). Site-directed mutagenesis of cytochrome c shows that an invariant Phe is not essential for function. Nature 313, 152-154.
21. Liang, N., Pielak, G.J., Mauk, A.G., Smith, M. and Hoffman, B.M. (1987). Yeast cytochrome c with phenylalanine or tyrosine at position 87 transfers electrons to (zinc cytochrome c peroxidase)$^+$ at a rate ten thousand times that of the serine-87 or glycine-87 variants. Proc. Natl. Acad. Sci. U.S.A. 84, 1249-1252.
22. Pielak, G.J., Oikawa, K., Mauk, A.G., Smith, M. and Kay, C.M. (1986). Elimination of the negative Soret Cotton effect of cytochrome c by replacement of the invariant phenylalanine using site-directed mutagenesis. J. Am. Chem. Soc. 108, 2724-2727.