The Catalytic Activity of Human Myoglobin is Enhanced by a Single Active Site Mutation: F43Y

David C. Levinger, Julie-Anne Stevenson and Luet-Lok Wong*†

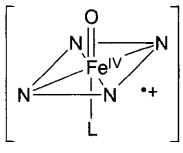
Inorganic Chemistry Laboratory, South Parks Road, Oxford, UK OX1 3QR

Phenylalanine-43 in the haem pocket of human myoglobin has been replaced by tyrosine using site-directed mutagenesis: the tyrosine-43 mutant is approximately 25 times more active than the wild-type protein in mediating the oxidation of styrene by hydrogen peroxide, and gives a 96:4 ratio of *R/S* styrene oxide (60% of products) in contrast to the racemate (46% of products) produced by the wild-type.

Haem proteins carry out a wide variety of functions in living organisms. Of particular significance are myoglobins and haemoglobins in the storage and transport of oxygen[1] respectively and oxidases, peroxidases and P450 enzymes in oxidative functionalisation.[2] Understanding the role of amino acids in creating an active-site environment that determines the catalytic activities of haem proteins is of fundamental importance, both in biological systems and in the design of synthetic molecular analogues for the selective oxidation of desired substrates.

Treatment of haemoglobin, myoglobin,[3] peroxidases, monooxygenases and catalases[4] with hydrogen peroxide gives ferryl intermediate **A**, [iron(IV)-oxo, porphyrin radical cations],[5] which is crucial in haem enzyme activity. In peroxidases, the ferryl species is reduced sequentially by two electrons to the ferric resting state, while in monooxygenase systems it attacks a substrate C–H bond leading to the formation of an alcohol functionality. The myoglobin ferryl intermediate, however, is very unstable and oxidises an amino acid side chain in the haem pocket.[6] This side chain radical in turn oxidises a tyrosine residue on the surface, leading ultimately to protein dimerisation.[7]

A number of studies have used myoglobin as a model to investigate factors that promote haem enzyme activity. Replacement of the surface tyrosines by phenylalanine gave mutant myoglobins which were more stable to dimerisation but not more active in mediating the oxidation of styrene by hydrogen peroxide.[8] Substitution of the haem axial ligand, histidine, with more electron-releasing residues such as cysteine and tyrosine resulted in mutant proteins which were at best five times more active than the wild-type protein in mediating styrene oxidation.[9] Here we report the results of a different approach. Phenylalanine-43 is on the distal side of the haem. The phenyl group of the side chain is nearly parallel to, and in van der Waals contact with (plane-to-plane distance *ca.* 4.0 Å),[1] the haem. By replacing phenylalanine-43 (F43) with tyrosine (Y), the myoglobin ferryl could oxidise the electron rich phenolic side chain to give a stable tyrosyl radical cation, and thus the second oxidising equivalent in the mutant could be retained in van der Waals contact with the haem.



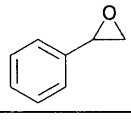
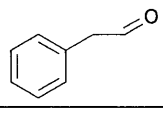
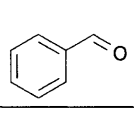
The ferryl intermediate **A**

The procedures for mutagenesis, and synthesis, refolding and purification of the recombinant proteins were as described elsewhere.[10] The thermal stabilities of the wild-type and F43Y proteins were very similar. The electronic spectra of the met forms of both proteins showed Sorét bands at 410 nm, corresponding to the presence of six-coordinate high-spin ferrihaems. There were differences in the visible region, resulting in the different colours of the wild-type (brown) and mutant (green). The spectra of the ferrous deoxy, oxy and carbonmonoxy forms were virtually identical, although the ferrous-oxy complex of the mutant was more susceptible to autooxidation. Computer modelling of the mutation based on the wild-type crystal structure suggested that, without significant backbone distortions, the distance between the phenolic oxygen of the tyrosine-43 and the haem iron would be too great for direct interaction.

Treatment of both wild-type and F43Y myoglobins with hydrogen peroxide gave the characteristic spectrum of the Fe^{IV}=O species (*i.e.* species **A** but in which the porphyrin ring is not oxidised) with bands at 421 and 545 nm.[3] This spectrum did not change after 2 h at 4 °C. The proteins were tested for their catalytic activities towards the oxidation of styrene by hydrogen peroxide. Analysis of the incubations by gas chromatography showed the products to be styrene oxide, phenylacetaldehyde, benzaldehyde and one unidentified product. No decomposition of styrene oxide was observed under the chromatographic conditions employed in this work. The product distribution and activity data are summarised in Table 1.

The reactions were not significantly affected by the presence of the chelating agent diethylenetriaminepentaacetic acid (DETAPAC) or mannitol, indicating that neither free ferric ions nor hydroxyl radicals were involved in the oxidations. The activity of both wild-type and F43Y mutant myoglobins increased with styrene and peroxide concentrations. The mutant

Table 1 The oxidation of styrene by hydrogen peroxide catalysed by wild-type and F43Y myoglobin[a]

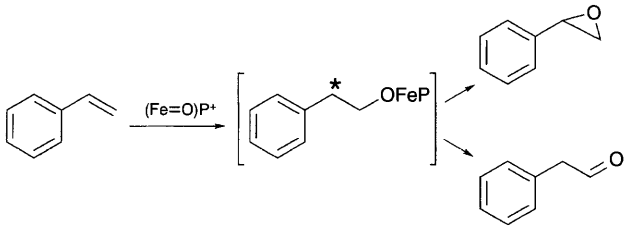
	Products formed and catalytic activity[b]				
				Other (unidentified)	Total turnover
Wild-type	46%	19%	19%	16%	(100%)
pmol/nmol WT	64	27	27	22	140
F43Y mutant	60%	29%	7%	4%	(100%)
pmol/nmol Y43	2260	1090	260	150	3760

[a] The reaction mixtures (1.0 ml total volume) contained the protein (150 μmol dm^{-3}) and styrene (10 mmol dm^{-3}). After pre-incubation at 0 °C for 5 min, hydrogen peroxide was added to a final concentration of 10 mmol dm^{-3} and the mixtures were incubated at 0 °C for 5 min. The reactions were quenched by extraction with 100 μl of chloroform and gas chromatographic analysis of the products performed using either a DB-1 (80 °C isotherm) or a chirality sensitive CDX-B column (100 °C isotherm). [b] The activities are expressed as pmol of product formed per nmol of haem in a 5 min. incubation.

was the more active at all concentrations, at 10 mmol dm^{-3} peroxide being approximately 25 times more active (Table 1). The stereospecificity of styrene epoxidation was analysed using a chiral phase GC column. In agreement with previous reports, the wild-type myoglobin gave a racemic mixture of (*R*)- and (*S*)-styrene oxide.[9,11] The reaction products of the F43Y mutant consisted of 60% styrene oxide with the *R*- and *S*-enantiomers (96:4).

The much higher catalytic activity of the F43Y mutant compared to the wild-type myoglobin indicates the success of the strategy of using a readily oxidised phenolic side chain to retain the oxidising equivalent close to the haem. In this respect the tyrosine-43 residue may be considered analogous to tryptophan-191 in cytochrome c peroxidase, in which the reaction of the ferric enzyme with peroxide results in an iron(IV)-oxo species and the W191 side chain is oxidised to a radical cation.[12]

The proposed mechanism of haem protein-catalysed styrene oxidation is shown in Scheme 1.[2] Oxidation of the olefinic double bond gives rise initially to a carbon-based radical or cation intermediate, which then either collapses to give the epoxide, or undergoes hydrogen–proton migration to give the aldehyde. For enantiospecific epoxidation to occur, the styrene must be bound such that only one side of the double bond is attacked by the iron-oxo group, and there is no C–C bond rotation in the three-coordinate carbon intermediate. The formation of a racemic mixture of styrene oxide in the reaction mediated by the wild-type human myoglobin is consistent with the oxidation occurring on the protein surface rather than in the haem pocket.[11] In contrast, the enantiospecific epoxidation of sytene to (*R*)-styrene oxide by F43Y myoglobin strongly suggests that styrene oxidation occurs in the haem pocket of the mutant and thus highlights a change in mechanism as compared with the wild-type.



Scheme 1 A proposed mechanism of styrene oxidation by a ferryl intermediate. The asterisk denotes the three coordinate radical or carbocation centre.

In conclusion, a single mutation introducing an amino acid side chain that can form a more stable cation into the haem pocket has dramatically increased the catalytic activity of human myoglobin, and styrene epoxidation catalysed by the mutant protein is enantiospecific. These observations suggest that the catalytic activity of haem model compounds could also be modified by introducing electron-rich groups in the immediate vicinity of the haem iron.

We thank the Royal Society and the Nuffield Foundation for support of this work, and Professor S. G. Boxer for a generous gift of the human myoglobin clone and expression system. D. C. L. wishes to thank the BBSRC for a studentship.

Received, 7th August 1995; Com. 5/05286F

Footnote

† E-mail: LLW@VAX.OX.AC.UK

References

1 *Haemoglobin and Myoglobin in Their Reactions with Ligands*, E. Antonini and M. Brunori, North-Holland, Amsterdam, 1971.
2 *Cytochrome P450, Structure, Mechanism and Biochemistry*, ed. P. R. Ortiz de Montellano, Plenum Press, New York, 1986.
3 P. Geroge and D. H. Irvine, *Biochem. J.*, 1952, **52**, 511; N. K. King and M. E. Winfield, *J. Biol. Chem.*, 1963, **238**, 1520.
4 L. J. Marnett, P. Weller and J. Battista, in ref. 2.
5 H. B. Dunford, *Adv. Inorg. Biochem.*, 1982, **4**, 41.
6 N. K. King, F. D. Looney and M. E. Winfield, *Biochem. Biophys. Acta*, 1967, **133**, 65; T. Yonetani and H. Schleyer, *J. Biol. Chem.*, 1967, **242**, 1974.
7 D. Tew and P. R. Ortiz de Montellano, *J. Biol. Chem.*, 1988, **263**, 17880.
8 S. I. Rao, A. Wilks and P. R. Ortiz de Montellano, *J. Biol. Chem.*, 1993, **268**, 803.
9 S. Adachi, S. Nagano, K. Ishimori, Y. Watanabe, I. Morishima, T. Egawa, T. Kitagawa and R. Makino, *Biochemistry*, 1993, **32**, 241.
10 R. Varadarajan, A. Szabo and S. G. Boxer, *Proc. Natl. Acad. Sci. USA*, 1985, **82**, 5681.
11 P. R. Ortiz de Montellano and C. E. Catalano, *J. Biol. Chem.*, 1985, **16**, 9265.
12 M. Sivaraja, D. B. Goodin, M. Smith and B. M. Hoffman, *Science*, 1989, **245**, 738.