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Amino acid sequence, haem-iron co-ordination geometry and functional properties of mitochondrial and bacterial c-type cytochromes

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1. Introduction

Cytochromes are found in all biological oxidation systems which involve transport of reducing equivalents through organized chains of membrane bound intermediates, regardless of the ultimate oxidant (Keilin, 1966; Bartsch, 1978; Meyer & Kamen, 1982). Thus, cytochromes are present not only in the aerobic mitochondrial and bacterial respiratory chain, but are also found in much more diversified procariotic systems, including all varieties of facultative anaerobes (nitrate and nitrite reducers), obligate anaerobes (sulphate reducers and phototrophic sulphur bacteria), facultative photoheterotrophes (phototrophic non-sulphur purple bacteria), and the photoautotrophic cyanobacteria (blue-green algae). Among the different types of cytochromes occurring in the cell, the soluble c-type cytochromes ('class I', Meyer & Kamen, 1982) are the most abundant and best characterized group of proteins (Bartsch, 1978; Meyer & Kamen, 1982; Dickerson & Timkovitch, 1975; Lemberg & Barrett, 1973; Salemme, 1977; Ferguson-Miller, Brautigan & Margoliash, 1979). The amino acid sequences of more than 80 mitochrondrial and close to 40 bacterial cytochromes c are known (Meyer & Kamen, 1982; Dickerson & Timkovitch, 1975; Schwartz & Dayhoff, 1976; Dayhoff & Barker, 1978).

A search for the biochemical rational behind the persistent occurrence of histidine and methionine as axial ligands of the haem-iron has been our motivation for systematic studies of correlations between the active site conformation and the primary structure, the electronic structure of haem c and functional properties, such as the redox potential or the reactivity with cytochrome oxidases and reductases from different species. The omnipresence of soluble c-type cytochromes in nature has already stimulated many comparative structural (Dickerson & Timkovitch, 1975; Timkovitch, 1979; Moore *et al.* 1982), evolutionary (Meyer & Kamen, 1982; Dickerson, 1980*a*, *b*, *c*) and functional studies (Ferguson-Miller *et al.* 1979; Errede & Kamen, 1978; Yamanaka & Okunuki, 1968; Sutin, 1977). Thereby the characterization of the protein surface responsible for the interaction with physiological redox-partners has been the target of numerous recent biochemical investigations (Ferguson-Miller *et al.* 1979; Rieder & Bosshard, 1980; Waldmeyer *et al.* 1982; Kraut, 1981). In contrast, there have been few comparative studies of the structure of the active centre of cytochromes c. This centre lies in the interior of the protein and consists of the haem group and two axial

TABLE 1. *Comparison of the amino acid sequences of selected mitochondrial and bacterial cytochromes c for which the haem-iron co-ordination geometry was determined by ^1H NMR in solution. The sequences are aligned as suggested by Dickerson (1980a, c). Dashes indicate gaps in the alignment. The numbering along the top of the table is that of the horse sequence. The haem group is covalently bound to Cys-14 and Cys-17, with the exception of cytochrome c-557 from* C. oncopelti *where the haem is covalently linked only to Cys-17. The haem-iron is ligated by His-18 and Met-80. A box is drawn around that region of the sequence for which a correlation with the chirality of the axial methionine is indicated by the presently available data.*

	−10	1	10	20	30	40	50	60	70	80	90	100
Horse		GDVEKGKKI	FVQK-CAQCHT	VEKGGKHKTG	PNLHGLFGRK	TGQAPGFTYT	DANKN---KGITW	KEETLMEYLE	NPKKYIP---------G-TK	M----IFAG-IKK-KT	EREDLIAYLK	KATNE
C. krusei	PAPFEQ	GSAKKGATL	FKTR-CAQCHT	IEAGGPHKVG	PNLHGIFSRH	SGQAEGYSYT	DANKR---AGVEW	AEPTMSDYLE	NPKKYIP---------G-TK	M----AFGG-LKK-AK	DRNDLVTYML	EASK
S. cerevisiae Iso-1	TEFKA	GSAKKGATL	FKTR-CLQCHT	VEKGGPHKVG	PNLHGIFGRH	SGQAEGYSYT	DANIK---KNVLW	DENNMSEYLT	NPKKYIP---------G-TK	M----AFGG-LKK-EK	DRNDLITYLK	KACE
S. cerevisiae Iso-2	AKESTGFKP	GSAKKGATL	FKTR-CQQCHT	IEEGGPNKVG	PNLHGIFGRH	SGQVKGYSYT	DANIN---KNVLW	DEDSMSEYLT	NPKKYIP---------G-TK	M----AFAG-LKK-EK	DRNDLITYMT	KAAK
C. oncopelti c-557	GPKAREPLPP	GDAAKGEKI	FKGR-AAQCHT	GAKGGANGVG	PNLFGIVNRH	SGTVEGFAYS	KANAD---SGVVW	TPEVLDVYLE	NPKKFMP---------G-TK	M----SFAG-IKK-PQ	ERADLIAYLE	NLK
R. rubrum c_2	E	GDAAAGEKV	SKK--CLACHT	FDQGGANKVG	PNLFGVFENT	AAHKDNYAYS	ESYTEMKAKGLTW	TEANLAAYVK	DPKAFVLEKSGDPK--AKSK	M----TFK--LTK-DD	EIENVIAYLK	TLK
E. gracilis c-552		GGADV	F-ADNCSTCHV	NG---GNVIS	AG-KVL-SKT	AIE-------	--------EYLDG	GY-TKE-AIE	-YQVRN----------GKGP	M---PAWEGVLS--ED	EIVAVTDYVY	TQAGGAWANV
S. maxima c-553		GDVAAGASV	F-SANCAACHM	GG---RNVIV	AN-KTL-SKS	DLAK------	--------YLKGF	DDDAVA-AVA	-YQVTN----------GKNA	M---PGFNGRLS--PL	QIEDVAAYVV	DQAEKGW
Rps. gelatinosa c-551		ATPAEL	ATKAGCAVCHQ	PTA--K-GLG	PSYQEIAKKY	K---------	---------GQAG	APALMAERVR	KGSVGIF---------GKLP	MTPTPPAR--IS--DA	DLKLVIDWIL	KTP
P. mendocina c-551		ASGEEL	FKSKPCGACHS	VGA--K-LVG	PALKDVAAKN	A---------	---------GVDG	AADVLAGHIK	NGSTGVW---------GAMP	M---PPNP--VT--EE	EAKTLAEWVL	TLK
P. aeruginosa c-551		EDPEVL	FKNKGCVACHA	IDT--K-MVG	PAYKDVAAKF	A---------	---------GQAG	AEAELAQRIK	NGSQGVW---------GPIP	M---PPNA--VS--DD	EAQTLAKWVL	SQK
P. stutzeri c-551		QDGEAL	FKSKPCAACHS	IDA--K-LVG	PAFKEVAAKY	A---------	---------GQDG	AADLLAGHIK	NGSQGVW---------GPIP	M---PPNP--VT--EE	EAKILAEWIL	SQK
C. limicola c-555		YDAAAGKAT	YDAS-CAMCHX	TGM----MGA	PKVGDKAA--	----------	---------WAPH	IAKGMNVMVA	NSIKGYKG---------TKGM	M---PAKGGNPKLTDA	QVGNAVAYMV	GQSK
P. mendocina c_5	AASAG	GGARSADDI	I-AKHCNACHG	AGV----LGA	PKIGDTAA--	----------	---------WKER	AD---HQGGL	DGILAKIS--------GINA	M---PPKGTCAD--CS	DDELREAIQK	MSGL
D. vulgaris c-553		ADGAAL	Y--KSCIGCHS	ADG----GKA	MMTNAVKG--	----------	---------KYSD	EELKALADYM	KAAMGSAKPVKGQGAEELYK	M---KGYADGSY--GG	ERKAMSKL	

ligands of the haem-iron, i.e. histidine and methionine (Dickerson & Timkovitch, 1975; Wüthrich, 1969). In all known cytochrome c sequences the axial histidine and methionine (His 18 and Met 80 in Table 1) are the only strictly conserved amino-acid residues in the primary structure (Meyer & Kamen, 1982; Schwartz & Dayhoff, 1976; Dayhoff & Barker, 1978) besides Cys 17.

The present paper surveys the available data on the haem-iron co-ordination geometry in class I cytochromes (Senn, 1983; Senn & Wüthrich, 1983*a*, *b*, *c*; Senn, Keller & Wüthrich, 1980; Senn, Eugster & Wüthrich, 1983*a*; Senn *et al.* 1983*b*; Senn, Billiter & Wüthrich, 1984*a*; Senn, Böhme & Wüthrich, 1984*b*; Senn, Cusanovich & Wüthrich, 1984*c*; Keller, Picot & Wüthrich, 1979, 1980; Ulrich, Krogmann & Markley, 1982; Salemme *et al.* 1973; Takano & Dickerson, 1981*a*, *b*; Matsuura, Takano & Dickerson, 1982; Timkovich 1979) and investigates possible correlations with the amino acid sequence and functional properties. The proteins studied (Table 1) were selected from a broad range of eucaryotic and bacterial organisms so that the investigations could be extended to phylogenetic information on the haem-iron co-ordination geometry and the haem c electronic structure.

2. Survey of the co-ordination geometry of the two haem-iron axial ligands histidine and methionine in ferrocytochromes c

(*a*) *The axial methionine*

Four different types of methionine co-ordination geometries in the c-type cytochromes investigated (Table 1) have been characterized in solution, and two of these have also been observed in cytochrome c crystal structures (Fig. 1). In cytochromes c from mammalian species (horse and tuna) (Senn *et al.* 1980, 1984*a*; Takano & Dickerson, 1981*a*), and from yeast (*S. cerevisiae*, *C. krusei*) (Senn *et al.* 1983*a*), cytochrome c-557 from *C. oncopelti* (Keller *et al.* 1979) cytochromes c-552 and c-553 from *E. gracilis*, *S. platensis* and *S. maxima* (Keller, Schejter & Wüthrich, 1980; Senn *et al.* 1984*b*, Ulrich *et al.* 1981) and cytochrome c_2 from *Rhodospirillum rubrum* (Senn & Wüthrich, 1983*b*; Salemme *et al.* 1973) the axial methionine has R chirality at the iron-bound sulphur and the methionine side-chain is extended, with the C^{α} carbon near the β-meso position of haem c and outside the porphyrin ring skeleton (Fig. 1A). In the cytochromes c-551 from *P. aeruginosa* (Senn *et al.* 1980, 1984*c*;

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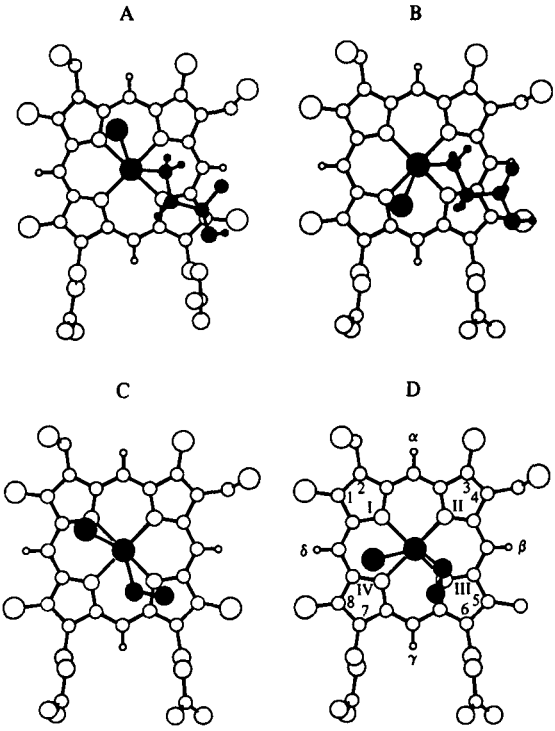

Fig. 1. Conformation of the axial methionine in selected mitochondrial and bacterial c-type cytochromes. The view is perpendicular to the heme plane. The methyl and methylene groups are represented by equivalent spheres M and L, with the exception of $C^{\beta}H_2$ and $C^{\gamma}H_2$ of methionine in the structures A and B, where these methylene protons were stereospecifically assigned (Senn *et al.* 1984*a*). (A) Mitochondrial ferrocytochromes c (*S. cerevisiae* Iso-1 and Iso-2, *C. krusei*, *C. oncopelti*, horse), *R. Rubrum* cytochrome c_2, *S. platensis* ferrocytochrome c-553 and *E. gracilis* ferrocytochrome c-552. (B) Ferrocytochromes c-551 of *P. aeruginosa*, *P. mendocina*, *P. stutzeri* and *Rps. gelatinosa*. (C) *P. mendocina* ferrocytochrome c_5. $C^{\alpha}H$ is not shown (see text). (D) *D. vulgaris* and *D. desulfuricans* ferrocytochromes c-553. In structure D the meso-positions and the β positions of haem c are identified by α–δ, and by 1–8, respectively. The pyrrole rings are numbered I–IV.

Matsuura *et al.* 1982), *P. mendocina* (Senn & Wüthrich, 1983*b*), *P. stutzeri* (Senn & Wüthrich, 1983*b*) and *Rps. gelatinosa* (Senn & Wüthrich, 1983*a*) the axial methionine has S chirality at the iron-bound sulphur and the methionine side chain is bent, so that $C^{\beta}H_2$ is closer to the ϵ-methyl group than $C^{\gamma}H_2$. $C^{\alpha}H$ is near pyrrole ring III and outside the porphyrin ring skeleton (Fig. 1 B). In cytochrome c_5 from *P. mendocina* the axial methionine has S chirality, the methionine side chain is extended and $C^{\beta}H_2$ is located above the

pyrrole ring III (Fig. 1 C). $C^{\alpha}H$ is not shown in Fig. 1 C, but observations on its chemical shift indicate that it is near the haem-plane and within the confines of the porphyrin ring (Senn & Wüthrich, 1983*c*). The structure for the ferrocytochromes c-553 from *Desulfovibrio vulgaris* and *Desulfovibrio desulfuricans* (Fig. 1 D) is most closely related to that found in cytochromes c-551 (Fig. 1 B) (Senn *et al.* 1983*b*). It coincides with the latter in the S chirality at the iron-bound sulphur and the bent conformation of the $S\text{-}C^{\gamma}H_2\text{-}C^{\beta}H_2$ fragment. It differs from the cytochrome c-551 structure by a clockwise rotation by approx. 45° of the methionine about the iron-sulphur bond. Furthermore, it is so far a unique feature of the two ferrocytochromes c-553 investigated that the methionine $C^{\gamma}\text{-}C^{\beta}$ bond is directed away from haem-plane (Fig. 1 D). In all other cytochromes c (Fig. 1 A–C) both methylene groups of the axial methionine adopt an orientation in which one proton points towards and the other points away from the haem-plane.

(*b*) *The axial histidine*

The same spatial arrangement of the axial histidine prevails in the conformations of all cytochromes c investigated so far in solution and in single crystals (Timkovitch, 1979) (Table 1): the imidazole ring plane is oriented approximately along a line through the meso-protons α and γ and is roughly perpendicular to the haem-plane (see Fig. 1 D for haem nomenclature).

(*c*) *Co-ordination geometry in ferricytochromes c*

The chirality of the axial methionine binding to the haem-iron in the ferric state of the proteins is accessible for investigation by X-ray methods and CD-spectroscopy (Senn *et al.* 1980). With the exception of the two *Desulfovibrio* cytochromes c-553 (Senn *et al.* 1983*b*) all cytochromes c of Table 1 show identical chirality at the axial methionine-sulphur in the oxidized and reduced state (Table 2). In *Desulfovibrio* cytochromes c-553 different methionine chirality was observed in the two oxidation states of the same protein, i.e. in the reduced ferro- and in the oxidized ferri-form.

TABLE 2. *Chemical shifts of the haem ring methyl* ^1H *NMR lines in the ferric state, chirality of the axial methionine and oxidation-reduction potentials of the cytochromes c in Table 1. In the first column the numbers indicate the resonance positions of the ring methyls at 35 °C (see Fig. 1D for nomenclature used) with respect to the chemical shift scale at the bottom of the table. The third column lists the chirality of the axial methionine sulphur in the oxidized and reduced state of the proteins, R or S (Keller* et al. *1980; Senn & Wüthrich 1983*a, b, c; *Senn* et al. *1980, 1983*a, b, *1984*a, b, c). *The fourth column lists literature data on the oxidation-reduction potentials in mV (Sugimura* et al. *1968; Lemberg & Barrett 1973; Bartsch 1978; Bertrand* et al. *1982; Meyer & Kamen 1982).*

Ring methyl chemical shifts	Species	Met. chirality Ox.	Met. chirality Red.	E' (mV)
8 3 5 1	Horse c	R	R	260
8 3 5 1	*C. krusei* c	R	R	260
8 3 5 1	*S. cerevisiae* c Iso-1	R	R	260
8 3 5 1	*S. cerevisiae* c Iso-2	R	R	260
8 3 5 1	*C. oncopelti* c-557	R	R	255
8 3 5 1	*R. rubrum* c_2	R	R	320
8 3 5 1	*E. gracilis* c-552*	R	R	325
5 1 8 3	*R. gelatinosa* c-551	S	S	280
5 1 8 3	*P. mendocina* c-551	S	S	~ 200
5 1 8 3	*P. aeruginosa* c-551	S	S	285
5 1 8 3	*P. stutzeri* c-551	S	S	280
8 5 3 1	*C. limicola* c-555	-†	-†	145
8 3 5 1	*P. mendocina* c_5	S	S	320
8 3 5 1	*D. vulgaris* c-553‡	R	S	0

50 40 30 20 10
ppm

* Identical features were observed for *S. platensis* cytochrome c-553 (a cyanobacterium), for which only the sequence of the N-terminal 44 residues is known (Senn *et al.* 1984*b*).

† In *C. limicola* cytochrome c-555 the co-ordination geometry of the axial methionine was not determined (Senn *et al.* 1984*c*).

‡ Identical heme co-ordination and oxidation-reduction potential prevail for *D. desulfuricans*, of which the amino acid sequence is not known (Senn *et al.* 1983*b*).

3. Amino acid sequence and stereo-selective ligand binding to the haem-iron

(*a*) *The axial methionine*

The stereospecificity of the axial methionine binding to the haem-iron cannot be correlated with the overall primary structure homology. For example *E. gracilis* cytochrome c-552 has 13 sequence positions in common with horse cytochrome c and 19 positions in common with *P. mendocina* cytochrome c-551 (Dickerson, 1980*c*). Nevertheless, the axial methionine sulphur atoms in both horse cytochrome c and *E. gracilis* cytochrome c-552 exhibit R chirality, whereas the sulphur atom in cytochrome c-551 exhibits S chirality. Among the mitochondrial and bacterial cytochromes c with identical stereospecificity of the methionine binding to the haem-iron (Table 2) amino acid sequence homology as low as 20% is observed. (Table 1, e.g. *E. gracilis* cytochrome c-552 and horse cytochrome c). From these data, we conclude that only local sequence segments are responsible for the stereoselective-methionine binding to the haem-iron.

One such segment was found in the immediate vicinity of the axial methionine (boxed region, Table 1).

All cytochromes c with S-chirality at the sulphur atom contain several prolines around the axial Met 80 (Table 1). The sequential order of these proline residues appears to be correlated with the stereospecific methionine binding. Proteins having two prolines in consecutive positions following Met 80 in the boxed region (Table 1) have S-chirality. However, if only one proline occurs after Met 80 in the primary structure, an R-chiral attachment of the methionine to the haem-iron is observed (e.g. *E. gracilis* and *S. maxima* cytochrome c-552, Table 1). This correlation does not apply for *Desulfovibrio* cytochromes c-553 (Table 1), which show an S-chiral axial methionine in the reduced and an R-chiral methionine in the oxidized form of the protein.

The aromatic amino acid at position 82 in the primary structure (Table 1) is observed in all cytochromes c with R-chiral axial methionine (M80) but is missing in the S-chiral co-ordination types.

In recent model studies of the interaction of palladium with S-methyl-cysteinyl peptides, local changes in the peptide sequence have been shown to affect the diastereomeric ratio of the two Pd-complexes formed (Kozlowski *et al.* 1983). From single crystal studies on the tertiary structure of mitochondrial cytochromes c and

R. rubrum cytochrome c_2, proteins with R-chiral methionine, it is known that the lone pair sp^3-orbital of the axial methionine sulphur is involved in an H-bond to tyrosine $67\,O_\eta$ (Table 1) (Takano & Dickerson, 1981*a*; Salemme *et al.* 1973). In the tertiary structure of *P. aeruginosa* cytochrome c-551, a protein with S-chiral methionine, N_δ of Asn in position 64 (corresponds to position 82 in horse cytochrome c Table 1) takes on the function as H-donor to the axial methionine sulphur lone pair orbital (Matsuura *et al.* 1982). The homologous position to residue Asn 64, however, is Phe 82 and not Tyr 67 in mitochondrial and *R. rubrum* cytochrome c_2. This results from the different spatial orientations of the axial methionine sulphur lone pair orbital in the two classes of proteins (Fig. 1 A and B). The residues in position 67 and 82 of the numeration used in Table 1 are conserved in most cytochromes c. In *S. maxima* cytochrome c-553 and *E. gracilis* cytochrome c-552, both proteins with R-chiral methionine, no amino acid with H-donor capacity homologous to Tyr 67 is found in the homologous sequence alignments of Table 1. Whether the alignments have to be corrected for these two proteins remains an open question as long as their tertiary structures are unknown.

The two remaining structural types, *P. mendocina* cytochrome c_5 (Fig. 1 C) and *Desulfovibrio* ferrocytochromes c-553 (Fig. 1 D) have S-chiral methionine attachment, but otherwise completely different methionine conformations than *Pseudomonas* cytochromes c-551 (Fig. 1 B). The H-donor amino acid to the axial sulphur atom can therefore not be localized in the primary sequence from a consideration of homology to the primary structure of *Pseudomonas* cytochromes c-551.

(*b*) *The axial histidine*

The same spatial arrangement of the axial histidine as observed by high-resolution NMR techniques in solution (Senn, 1983; Senn & Wüthrich 1983*a*, *b*, *c*; Senn *et al.* 1980, 1983*a*, *b*, 1984*b*, *c*) has also been observed in the crystalline state (Timkovitch 1979). An important feature in determining the axial histidine orientation appears to be the presence of an H-bond between the N_1H of the axial imidazole ring and the C=O group of Pro 30 (Takano & Dickerson, 1981*a*; Salemme *et al.* 1973; Matsuura *et al.* 1982) (Table 1). From the observation that the protein sequences of all mitochondrial and most of the bacterial c-type cytochromes show a conserved proline at position 30 (Table 1) (Bartsch 1978; Schwartz & Dayhoff, 1976;

Dayhoff & Barker, 1978; Dickerson, 1980*a*), we infer that this homologous position assists in conservation of the spatial orientation of the axial histidine.

4. Correlation between the co-ordination geometry of the axial methionine and other properties related to cytochrome c function

(*a*) *Axial methionine co-ordination geometryand electronic haem c structure*

Earlier work on the unpaired electron spin density distribution in the porphyrin ring of cytochromes c indicated a clear correlation between the asymmetry of the haem c electronic structure and the presence of a methionine ligand at the sixth co-ordination position of the haem-iron (Wüthrich, 1970, 1971). Later, the unpaired spin density could be assigned to individual pyrrole rings of haem c (Redfield & Gupta, 1971; Keller & Wüthrich, 1978*a*).

In the present study we have further investigated correlations between conformational properties of the axial ligand sphere and the haem c electronic structure. Table 2 reveals common traits as well as differences between the cytochromes c investigated. The chemical shifts of the individually assigned haem-ring methyl resonances reflect the delocalization of the unpaired electron of the low-spin ferric iron in the haem-plane (Wüthrich, 1970, 1976). In all species, there are two methyl groups attached to opposite pyrrole rings (see Fig. 1 D for nomenclature used) which experience large hyperfine shifts, wheras the other two ring methyls are shifted to a lesser extent. The large hyperfine shifts indicate that up to 3% of the unpaired electron spin density is localized in the π orbital of the β-ring carbon to which the methyl is attached (Fig. 1 D). The small hyperfine shifts correspond to an unpaired electron spin density of less than 0·5% in the corresponding β-carbon π orbital (Wüthrich, 1976). (Cytochrome c-555 in Table 2 is omitted from this discussion because its methionine structure has not been determined (see Senn *et al.* 1984*c* for a detailed discussion of this protein).)

The two classes of haem c electronic structure observed in cytochromes c are schematically shown in Fig. 2. In *Pseudomonas* cytochromes c-551 and in *Rps. gelatinosa* cytochrome c-551 the high spin density is at positions 1 and 5 on the pyrrole rings I and III (Fig. 2), whereas in all other cytochromes c investigated methyls 3 and 8 experience a larger shift, which manifests high spin density on the

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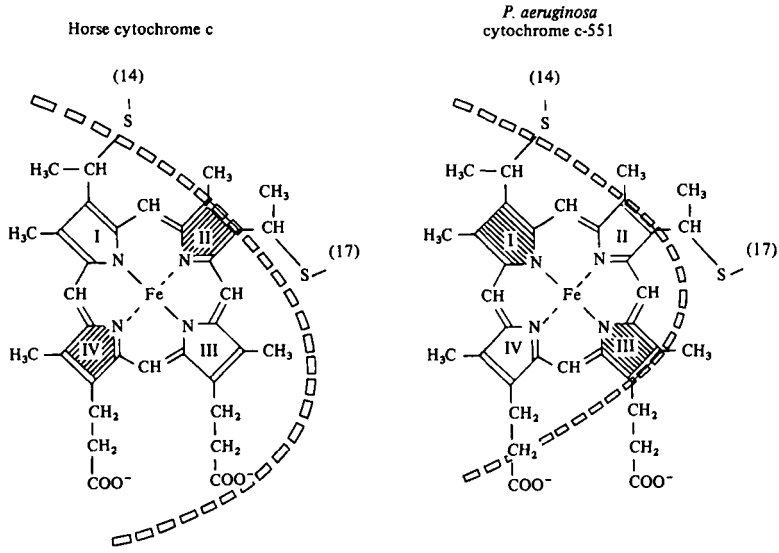

Fig. 2. Schematic representation of the electronic structure of haem c in horse ferricytochrome c and *P. aeruginosa* ferricytochrome c-551. The shaded pyrrole rings are those where high electron spin density (3–5 %) is observed on the β carbon atoms. The thick broken line indicates the protein surface. In both species the edge of the pyrrole ring II is accessible on the surface. Because of extensive deletions in the polypeptide chain of *P. aeruginosa* cytochrome c-551 (Table 1) the edge of pyrrole ring III is also accessible on the protein surface of this species (Matsuura *et al.* 1982).

pyrrole rings II and IV. There is a strict correlation between the haem c electronic structure and the co-ordination geometry of the axial methionine (Table 2, Fig. 1). A likely explanation is suggested by the direct correspondance with the orientation of the lone pair electrons of the ligand methionine sulphur atom. The interaction of the sp^3 lone-pair electrons of the methionine sulphur with the d_{xz} and d_{yz} orbitals of the iron modifies the relative energies of the molecular orbital involving d_{xz} and d_{yz}, which results in a marked effect on the distribution of the unpaired electron spin density of the low spin ferric iron between these two orbitals (Senn *et al.* 1980; Shulman, Glarum & Karplus, 1971). In the structures of Fig. 1 A and B the lone pair is directed at the pyrrole nitrogens IV and I, respectively. As long as the change in methionine conformation is restricted to a transition from R chirality to S chirality at the sulphur atom, the ensuing variation of the electronic structure consists of a rotation of the principle axes of the electronic g-tensor by approx. 90° about an axis perpendicular to the haem-plane (Senn *et al.* 1980; Keller *et al.* 1980;

Keller & Wüthrich, 1978*b*). Concomitant with this rotation is a shift in the location of high spin density on the peripheral pyrrole β-carbon atoms, from the pyrrole rings II and IV in structure A to the pyrroles I and III in structure B (Figs. 1 and 2). In ferricytochrome c_5 of *P. mendocina* the lone pair orbital of the axial methionine sulphur is oriented along a line through the nitrogen atoms of the pyrrole rings II and IV (Fig. 1 C). This coincides with the situation in mitochondrial cytochromes c (Fig. 1 A), except that the lone pair points in opposite directions in these two cases. Ferricytochrome c_5 has, therefore, S chirality at the axial methionine sulphur, but electron spin delocalization of the type observed, for example, in horse cytochrome c (Table 2, Fig. 2).

In the two investigated *Desulfovibrio* ferricytochromes c-553, the assymetry of the spin density distribution is less pronounced than in mammalian ferricytochromes c (Table 2) (Senn *et al.* 1983*b*). Since the detailed conformation of the methionine in the oxidized protein has not been determined, it is then of interest that this would be expected in a structure differing from that in Fig. 1 D only by a change of the chirality at the iron-bound sulphur from S to R (Senn *et al.* 1983*b*). The lone pair of the methionine sulphur atom would then not be directed straight at a pyrrole nitrogen, but would point in a direction somewhere between the pyrrole nitrogen IV and the meso-proton δ. The molecular orbitals derived from the d_{xz} and d_{yz} atomic orbitals of the iron would thus both contain some admixture of the sulphur lone-pair orbital, hence quite similar hyperfine shifts for the four-ring methyl resonances would be anticipated. In all cytochromes c investigated the imidazole ring plane is oriented along a line through the meso-protons α and γ and is roughly perpendicular to the haem-plane. The interaction of the π-system of the imidazole ring with the d_{xz}- and d_{yz}-orbitals of the haem-iron is symmetric in this orientation and does not change the relative energies of those haem-molecular orbitals which arise from admixture with iron atomic orbitals. It is therefore not surprising that an almost symmetric unpaired electron spin distribution has been observed in Azido-ferricytochrome c and cyanoferri-cytochrome c (Wüthrich, 1969; Gupta & Redfield, 1970), where the second axial ligand does not impose a pronounced asymmetry.

(*b*) *Axial methionine co-ordination geometry and redox potential*

The redox potential is the fundamental thermodynamic property of an electron transfer protein. It provides the basis to locate the protein

within an electron transport sequence. In order to understand mechanistic aspects of electron transfer reactions in proteins the structural basis for the control of the redox-potential has to be elucidated (Marcus, 1956; Hopfield, 1974; Jortner, 1976; Sutin, 1977; DeVault, 1980).

The redox potentials of the cytochromes c investigated vary over a wide range from approx. 350 mV for photosynthetic bacterial cytochromes c (Goldkorn & Scheijter, 1976; Yamanaka, Fukumori & Wada, 1978; Böhme *et al.* 1980) to approx. 0 mV for *Desulfovibrio* cytochromes c-553 (Table 2) (Bertrand *et al.* 1982). However, in eucariotic cytochromes c the heat and entropy of reaction corresponding to the redox couples have been highly conserved during phylogenetic evolution (Margalit & Schejter, 1973; Dickerson & Timkovitch, 1975; Pettigrew, Aviram & Schejter, 1975) and the redox potentials observed for mitochrondrial cytochromes c are all close to 260 mV (Table 2).

Several theories and hypotheses have been proposed to explain the structural bases responsible for the control of the redox properties in c-type cytochromes. These include the asymmetric distribution of electron density over the haem (Redfield & Gupta, 1971), variations in the hydrophobic environment of haem c (Kassner, 1972; 1973), different degrees of exposure of the haem edge to solvent (Stellwagen, 1978), differences in the length of the iron-sulphur bond (Moore & Williams, 1977), differences in the orientation of the axial histidine with respect to the haem-plane (Korszun *et al.* 1982), changes in the H-bond geometry of the axial histidine (His 18 N_1H-Pro30 CO) (Valentine *et al.* 1979) and differences associated with the charge on the haem propionates (Moore, 1983). Experimental observations (Kassner, 1972; Mashiko *et al.* 1981) and theoretical considerations (Kassner, 1973) have shown that the high redox potentials observed for cytochromes c, relative to model haem compounds with identical axial ligands in aqueous solution, are mainly due to the hydrophobic environment of haem c in the interior of the protein. However, the variations in redox potentials between different species (Table 2) cannot be rationalized with any of these hypotheses. The available data on c-type cytochrome structures do not conclusively support any of the proposed theories and are, in most cases, even contradictory (Korszun & Salemme, 1977; Fiechtner & Kassner, 1978; Pettigrew *et al.* 1978; Mashiko *et al.* 1981; Takano & Dickerson, 1981*a*; Korszun *et al.* 1982). Experimental results from the comparative structural studies of the active site conformation in c-type cytochromes

indicate a possible control mechanism for the redox properties in extreme low redox potential *Desulfovibrio* cytochromes c-553. In the *Desulfovibrio vulgaris* and *D. desulfuricans* cytochromes c-553 a different chirality at the axial methionine sulphur was observed in the ferri- and fero-state of the haem-iron (Table 2) (Senn *et al.* 1983*b*). This intriguing correlation between low redox potential and electron transfer-coupled change in the haem-iron co-ordination geometry could explain the difference in redox potential observed in *Desulfovibrio* cytochromes c-553. From the difference in redox potential of approx. 250 mV between *Desulfovibrio* cytochromes c-553 and the other c-type cytochromes c in Table 2 which show no chirality change in the ligand sphere upon reduction, the free energy needed for this conformational change can be estimated to be approx. 5 kcal/mol, i.e. this compares to about twice the free energy of a H-bond in a protein.

The cause of the rearrangement of methionine conformation upon valency change of the haem-iron is unknown, but might be triggered by a charge effect on internal hydrogen bonds of the haem and its axial ligands. The methionine conformations observed in the two redox states are equilibrium states which result after the electron transfer has occurred. However, the scheme of redox potential control described above may also suggest a general mechanism for facilitated electron transfer *in vivo*. External forces such as strong interactions with the oxidase or the reductase might change the cytochrome c conformation. If the oxidized molecule, for example, were forced to adopt a ligand conformation similar to the reduced form, it would become more prone to accept an electron. The apparent free energy of electron transfer would be lowered as a result of this conformation change. Experimental support for the potential of c-type cytochromes to adopt such intermediate structures comes from single crystal X-ray studies (Takano & Dickerson, 1981*a*, *b*; Matsuura *et al.* 1982), chemical modification (Ferguson-Miller *et al.* 1979; Osheroff *et al.* 1979, 1980; Koppenol & Margoliash, 1982) and NMR studies (Moore *et al.* 1982; Senn *et al.* 1983*a*, 1984*b*; Keller & Wüthrich, 1981). These studies also show that the biologically interacting surface of the globular molecule lies close to the axial methionine and is conformationally rather flexible.

(*c*) *Axial methionine co-ordination and enzymatic activity*

The surface topology and charge distribution in cytochromes c have been recognized as important structural determinants for the function of the molecule within its specific enzyme system (Errede & Kamen,

TABLE 3. *Comparison of cytochrome c reaction rates with mitochondrial oxidase and reductase and with* Pseudomonas *oxidase. The rel. reaction rates are taken from published data (Horio, 1958; Yamanaka & Okunuki, 1968; Errede & Kamen, 1978; Meyer & Kamen, 1982)*

	Relative reaction rate in % with		
	Mitochondrial		Pseudomonas
Cytochromes	Oxidase	Reductase	Oxidase*
Mitochondrial			
horse	100	100	2·5
C. oncopelti	220	98	—
C. krusei	70	—	5·0
Bacterial photosynthetic			
E. gracilis	0·05	3	8·5
S. maxima	0·05	2	—
R. rubrum	0·9	69	2·2
c-551 type			
P. aeruginosa	0·05	2	100
P. stutzeri	0	—	82
Rps. gelatinosa	0	0	—
C_5-type			
Pseudomonas	—	—	5

* Also named as cytochrome cd-nitrit reductase (Meyer & Kamen, 1982).

1978; Ferguson-Miller *et al.* 1979). Differences in the enzymatic activity of various chemically modified horse cytochromes could be quantitatively related to changes in the orientation of the electric dipole moments (Ferguson-Miller *et al.* 1979; Koppenol & Margoliash, 1982). However, differences in the reactivity between various eucaryotic cytochromes c or between mitochondrial and bacterial cytochromes c (Yamanaka & Okunuki, 1968; Errede & Kamen, 1978; Ferguson-Miller *et al.* 1979) are presently not understood on a structural basis. If we compare known relative enzymatic activities (Table 3) with structural features of the active site (Table 2), the following observations can be made:

All *Pseudomonas* cytochromes c-551 have high unpaired electron spin density on pyrrole rings II and IV of haem c (Table 2, Fig. 2) and show no enzymatic crossreactivity with the mitochondrial enzyme system, but high reactivity with its own oxidase.

All the other cytochromes c in Table 3 possess a horse type haem c

electronic structure (Fig. 2) and react poorly with the *Pseudomonas* cytochrome c oxidase.

There is no direct correlation of the enzymatic reactivity with the chirality of the axial methionine. This is clearly manifested in *Pseudomonas mendocina* cytochrome c_5, which does not react with *Pseudomonas* oxydase (Horio, 1958; Meyer & Kamen, 1982). Cytochrome c_5 has S-chiral methionine attachment to the haem-iron, as in all the cytochromes c-551, but its haem c electronic structure is of horse type due to the different orientation of its axial methionine (Fig. 1).

The asymmetric haem c electronic structure has previously been implicated in hypotheses concerning structure-function relationships in c-type cytochromes (Wüthrich, 1969; Redfield & Gupta, 1971). Based on the observation made above, a mechanism for facilitated electron transfer can be imagined. When cytochrome c binds to the enzyme, the binding interaction may be propagated from the surface to the axial methionine, which leads to an adjustment of its local conformation. The haem c electronic structure is thereby tuned to an optimal state in the productive complex. Experimental support for this hypothesis comes from enzymatic studies with various primate cytochromes c where a correlation has been found between productive complex formation and transition properties of the axial methionine sensitive absorption band at 695 nm (Osheroff *et al.* 1979). Further, the above hypothesis on the fine control of cytochrome c function are also based on the observation that the cytochrome c molecular structure is more flexible on the methionine side of the haem c (Keller & Wüthrich, 1981; Moore & Williams, 1980; Moore *et al.* 1982; Senn *et al.* 1983*a*, 1984*b*; Takano & Dickerson 1981*b*).

5. Phylogenesis of haem-iron co-ordination geometry and haem c electronic structure

Cytochromes c have been used extensively to examine relations of evolutionary variations in the protein structure to the phylogeny of the species (Dickerson & Timkovitch, 1975; Schwartz & Dayhoff 1976; Dayhoff & Barker, 1978; Meyer & Kamen, 1982; Dickerson, 1980*a*). The evolutionary trees constructed from similarities in primary and tertiary structures assume a common ancestor molecule for bacterial and mitochondrial cytochromes c (Schwartz & Dayhoff, 1976; Dayhoff & Barker, 1978; Dickerson, 1980*a*, *b*, *c*). However, the degree of divergence in the primary structure of cytochromes c is so

extensive that it has been argued that sequence variability has reached a limit which makes the constructions of such evolutionary trees questionable (Ambler *et al.* 1979*a*; Ambler, Meyer & Kamen, 1979*b*; Meyer & Kamen, 1982).

The available data show that in eucariotic cytochromes c the delocalization of the unpaired electronic spin in the haem-plane as well as the orientation of the electronic g-tensor have been conserved: the three yeast cytochromes c (*S. cerevisiae*, *C. krusei*), the protozoa cytochrome c-557 (*C. oncopelti*) and the mammalian cytochrome c have almost identical chemical shifts for the individual methyl and thiomethyl resonances of haem c (Table 2 (Keller & Wüthrich, 1978*a*;Keller *et al.* 1979; Senn *et al.* 1983*a*)). For tuna and turkey cytochromes c the same observation has also been made (Wüthrich, 1971; Moore & Williams, 1980). The invariant haem c electronic structure in the eucariotic proteins is clearly related to the strictly conserved haem-iron co-ordination geometry of the two axial ligands methionine and histidine (see Section 2). Thus, besides the electric dipole vector (Ferguson-Miller *et al.* 1979; Koppenol & Margoliash, 1982), the electronic and magnetic properties of the active centre have been conserved in eucariotic cytochromes c – even though approx. 50% of the amino acids differ in the amino acid sequences (Schwartz & Dayhoff, 1976; Dayhoff & Barker, 1978) during phylogenesis.

The bacterial cytochromes c for which data on the haem-iron co-ordination are available originate from aerobic, facultative anaerobic, strictly anaerobic and photosynthetic bacteria. These organisms show no close phylogenetic relationship to each other. In Tables 1 and 2 photosynthetic cytochromes are represented by *C. limicola* cytochrome c-555 (a green sulphur bacterium of the family *Chlorobiacea*), cytochrome c_2 from *Rhodospirillum rubrum* (a purple nonsulphur bacterium of the family *Rhodospirillaceae*), cytochrome c-553 from *Spirulina platensis* (a cyanobacterium) and chloroplast cytochrome c-552 from *Euglena gracilis*. All these proteins function in the phototrophic electron transport system. The specific function of cytochrome c-551 from the photosynthetic bacteria *Rhodopseudomonas gelatinosa* is unknown (Senn & Wüthrich, 1983*a*). The available data show that among the photosynthetic proteins, cytochrome c-552 from *E. gracilis* and cytochrome c-553 from *S. platensis* are most closely homologous to each other not only in the primary structure (Meyer & Kamen, 1982) but also in the co-ordination geometry and haem c electronic structure (Table 2, Fig. 1) (Senn *et al.* 1984*b*). This is in agreement with the close evolutionary relationship of the photo-

synthetic apparatus in the blue-green algae and in eucariotic algae, as expressed in the endosymbiotic theory (Marguilis, 1970). In contrast, the haem c electronic structure of *C. limicola* cytochrome c-555 is most similar to *P. mendocina* cytochrome c_5 (Senn & Wüthrich, 1983*c*; Senn *et al.* 1984*c*) which possesses a unique axial methionine structure (Fig. 1).

Phylogenetically, it is interesting that non-sulphur purple bacteria, cyanobacteria and chloroplasts contain c-type cytochromes with co-ordination geometries and haem c electronic structure closely homologous to mitochondrial cytochromes.

Denitrifying *Pseudomonas* bacteria contain cytochromes c-551 which exhibit a complete change in the active site conformation and haem c electronic structure when compared to eucaryotic species (Fig. 1 & 2, Table 2). They function as electron donors to the terminal oxidase, which in turn reduces molecular oxygen to water in aerobically grown cells and nitrite to N_2O in cells grown anaerobically on a nitrate medium (Meyer & Kamen, 1982).

The strictly anaerobic sulphate-reducing bacteria from *Desulfovibrio* species are adopted to a low redox potential environment. From the primary structure their cytochromes c-553 show some homology to eucariotic cytochromes c (Bruschi & LeGall, 1972). In the presence of formate, cytochrome c-553 can be reduced by the formate dehydrogenase of the same organism (Yagi, 1979). Among the species studied *Desulfovibrio* cytochromes c-553 are unique in the haem-iron co-ordination in the reduced form (Fig. 1) and by the fact that different methionine chirality prevails in the two oxidation states (Senn *et al.* 1983*b*).

A fourth type of co-ordination geometry for the axial methionine has been observed in *P. mendocina* cytochrome c_5 (Fig. 1 D). Since the amino acid sequence of cytochrome c_5 shows only very distant similarity to those of *Chlorobium limicola* cytochrome c-555 (Meyer & Kamen, 1982), and the functional role of cytochrome c_5 is still unknown, further study is required before an assessment can be made of how this unique cytochrome relates to inferences made here.

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