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Structural Bases for Function in Cytochromes *c*

AN INTERPRETATION OF COMPARATIVE X-RAY AND BIOCHEMICAL DATA*

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F. RAYMOND SALEMME,‡ JOSEPH KRAUT, AND M. D. KAMEN
From the Department of Chemistry, University of California, San Diego, La Jolla, California 92037

SUMMARY

The tertiary structures of the photosynthetic cytochrome c_2 of *Rhodospirillum rubrum* and eucaryotic mitochondrial cytochrome *c* are compared, together with data on their physiochemical properties and activities in physiological oxidoreduction systems. The comparison gives rise to the following observations and proposals.

1. *R. rubrum* cytochrome c_2 does not undergo the extensive oxidoreduction-linked conformation change characteristic of eucaryotic cytochromes *c*, but rather is structurally constrained in a conformation in both oxidation states which is over-all most similar to that of ferrocytochrome *c*.

2. Upon examination and comparison of the surface structural and charge topography of cytochromes *c* and c_2, it is apparent that they bear by far the closest similarity at their front sides (*i.e.* facing the heme crevice). This feature manifests itself clearly owing to the absolute invariance of many of the amino acid residues surrounding the perimeter of the heme crevice in both cytochrome c_2 and 41 species of eucaryotic cytochrome *c*.

3. A physiological mechanism for the oxidoreduction of eucaryotic cytochrome *c* is proposed which is in principle similar to that previously suggested for cytochrome c_2 (SALEMME, F. R., FREER, S. T., XUONG, NG. H., ALDEN, R. A., AND KRAUT, J. (1973) *J. Biol. Chem.* 248, 3910–3921). This mechanism involves front side interaction of the cytochrome *c* molecule with its oxidase and reductase, and direct electron addition to and withdrawal from the heme concomitant with destabilization of the existing heme oxidation state.

Progress toward an ultimate understanding of mechanisms whereby cytochromes *c* fulfill their various roles in coupled electron transport has been facilitated by the recent determination of the tertiary structures of cytochrome c_2 of the photosynthetic bacterium *Rhodospirillum rubrum* (1) and eucaryotic mitochondrial cytochrome *c* (2–4). The correlations which may be made between the structures, physicochemical properties, and physiological reactivities of these cytochromes are the subject of this report.

Eucaryotic mitochondrial cytochrome *c* has been shown both by sequential and structural studies (5) to be remarkably conservative in its evolution throughout both kingdoms of eucaryotes, implying that its functional role and concomitantly, its structural features, had been rigidly defined at the time of the emergence of the first multicellular organisms. These observations justify the assertion that cytochrome *c* is an "ancient" protein (2). Prior to the discovery of cytochrome c_2 from the photosynthetic bacterium *R. rubrum* (6), the occurrence of *c*-type cytochromes had been uniquely associated with eucaryotic organisms dependent upon oxidative phosphorylation for the production of ATP. Subsequent investigations have shown, however, that *c*-type cytochromes which are clearly related to eucaryotic mitochondrial cytochrome *c* are ubiquitous in nearly all living organisms (7), with the possible exception of some of the most primitive anaerobic and facultative microorganisms. Comparative study of the structure and function of the bacterial cytochrome c_2 of *R. rubrum*, a member of a group of organisms which is estimated to have differentiated from the eucaryotic ancestral line some 2×10^9 years prior to the emergence of the first multicellular organisms (8), with mitochondrial cytochrome *c* is consequently of interest both with regard to the early events in cytochrome *c* evolution and the evolution of phosphorylating electron transport chains.

Eucaryotic cytochrome *c* and cytochrome c_2 subserve analogous functions in their respective physiological electron transport chains; that is, they both transport electrons to the terminal and most oxidizing electron carrier of each system. Cytochrome c_2, by contrast with cytochrome *c*, has a somewhat more positive electrochemical potential, a nearly neutral isoelectric point, and does not exhibit the large oxidation-state dependent conformational change characteristic of mitochondrial cytochrome *c*. Table I briefly contrasts the properties of these 2 molecules. The following comparative analysis draws primarily on the previously published x-ray structure determinations of *R. rubrum* ferricytochrome c_2 (1), horse and bonito ferricytochrome *c* (3), and tuna ferrocytochrome *c* (4), which provide the structural bases for the correlation of physicochemical properties of these molecules.

METHODS

The Kendrew model of *R. rubrum* ferricytochrome c_2 based on the 2 A resolution multiple isomorphous replacement phased map

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‡ Present address, Department of Chemistry, University of Arizona, Tucson, Arizona 85721.

TABLE I

Comparative summary of properties of Rhodospirillum rubrum cytochrome c_2 and mitochondrial cytochrome c

Property	R. rubrum Cytochrome c_2	Mitochondrial Cytochrome c
Structure	112 residues + covalently bound Protoheme IX (MW = 12,840)	~104 residues + covalently bound Protoheme IX (MW = 11,800)
Physical Properties		
$E_{m,7}$	+ 320 mV	+ 260 mV
I.P.	6.3	10.1
	Does not undergo extensive conformation change on oxidoreduction	Undergoes extensive conformational change on oxidoreduction
Function	Photosynthetic electron transport chain	Mitochondrial oxidative phosphorylation electron transport chain
Reductase	ADP, ATP ⟷ Membrane bound cytochrome b	ADP, ATP ⟷ cytochrome b, cytochrome c_1 } reductase
	↓ cytochrome c_2 ↓	↓ cytochrome c ↓
Oxidase	ADP, ?, ATP ⟷ Membrane protein bound excited bacteriochlorophyll*	ADP, ATP ⟷ cytochrome a, cytochrome a_3 } oxidase
Location	30% bound to bacterial cell membrane, 70% soluble in cytoplasm	Bound to inner mitochondrial membrane or soluble in intramitochondrial space

was constructed as previously described (1). Accurate coordinates for the generation of stereo diagrams of cytochrome c_2 (Fig. 1) were obtained by use of an automated coordinate measuring device (9). Crystallographic refinement of cytochrome c_2 is the subject of another communication (10), but the important result for our present discussion is that no significant changes in the disposition of amino acid residues bounding the heme crevice were observed as compared to the model based upon the original multiple isomorphic-replacement phased map.

Coordinates for oxidized horse heart cytochrome c (2.8 A resolution) and reduced tuna heart cytochrome c (2.45 A resolution) were kindly furnished by R. Dickerson and T. Takano at the California Institute of Technology. The coordinates of the former structure were utilized for generation of stereo drawings (Fig. 2), and for accurate construction of a Kendrew model of horse ferricytochrome c. This model was constructed in an orientation best suited for comparison with the c_2 model, utilizing an automatic coordinate measuring device as described previously (9).

RESULTS

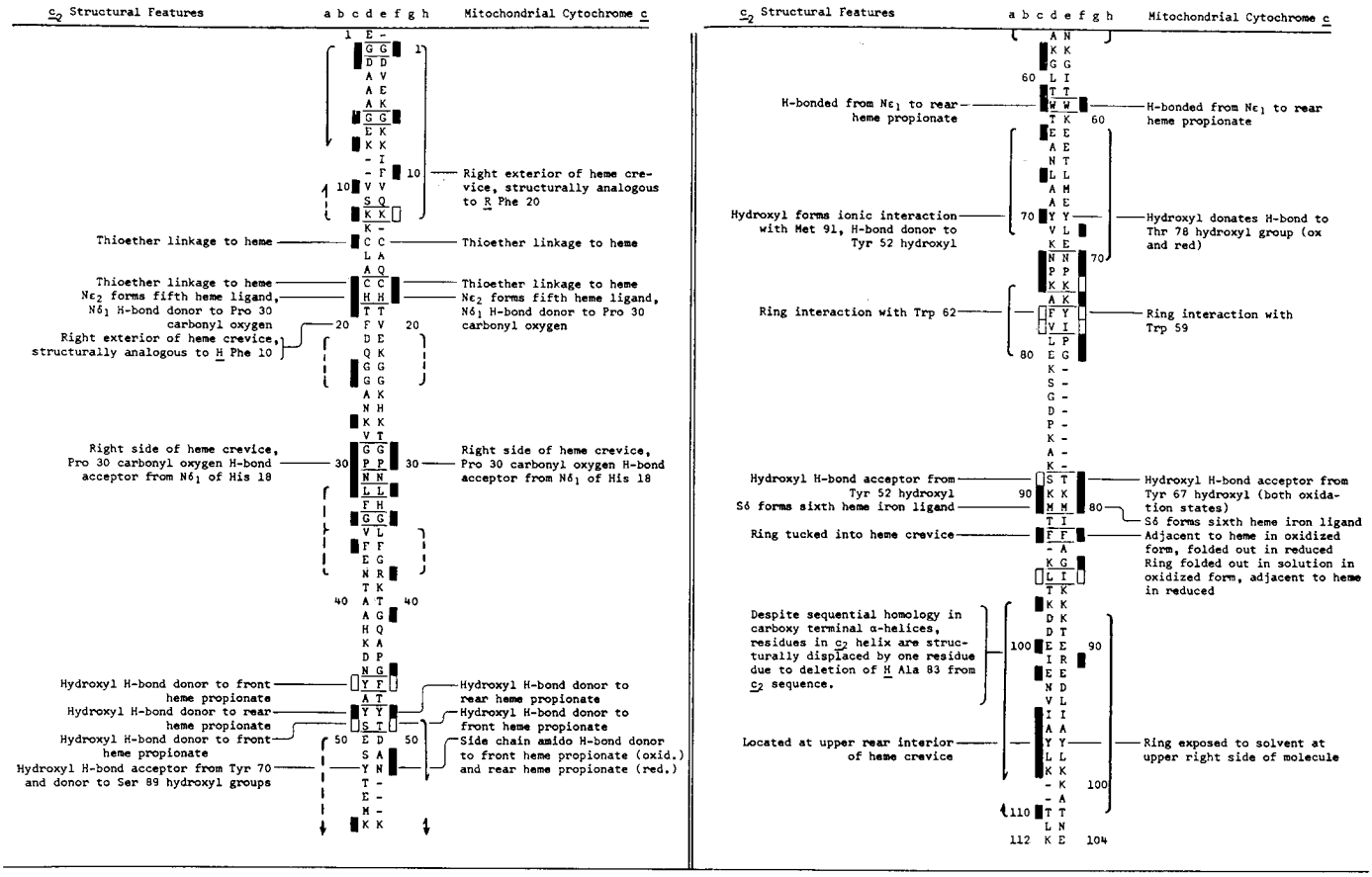
Structural and Sequential Homology—The sequential (11) and structural homology of cytochromes c_2 and c are compared and summarized in Table II. Figs. 1 and 2 show stereoscopic views of the ferricytochrome c_2 and c molecules from several equivalent aspects. It is readily seen that the tertiary structures of cytochromes c and c_2 are quite similar over-all, the principle conformational differences occurring at residue 53 where c_2 has a tripeptide insertion, and at residue *R*80-*H*78 where c_2 has an octapeptide insertion. (The abbreviations *R* and *H* are used hereafter to designate positions in the *R. rubrum* c_2 and horse cytochrome c sequences, respectively.) In addition, the c_2 sequence shows single residue insertions at positions *R*1 and 13, and several deletions corresponding to positions *H*9-10, 83, and 100-101 in the horse heart cytochrome c sequence. The deletion in the c_2 sequence corresponding to *H*9-10 appears to be structurally compensated by an insertion at *R*13 (see Table II), which results in essential similarity in the dispositions of the amino acid side chains in the corresponding regions of both molecules. The c_2 deletion at the position corresponding to *H*83, however, results in the structural displacement by one peptide unit of the residues comprising the c_2 COOH-terminal α helix (see Table II) relative to the analogous COOH-terminal α helix of horse heart cytochrome c. The dipeptide deletion from the c_2 sequence at positions corresponding to *H*100-101 does not manifest itself by any striking structural difference between c_2 and horse heart cytochrome c in this region. Rather, it appears that the dipeptide deletion structurally compensates for the one peptide unit displacement of the c_2 COOH-terminal α helix, relative to the cytochrome c terminal helix, such that *R* Thr 110 and *H* Thr 102 occupy approximately equivalent positions in both molecules. As can be seen from Figs. 1 and 2, and Table II (Columns a and h), both molecules contain structurally equivalent regions of α helical configuration at the NH_2 terminus and in the vicinity of *R*64-*H*61, in addition to the analogous COOH-terminal α helices described above. The α helix in cytochrome c beginning at *R*49 is approximated in c_2 by a series of type I_{10} loops (12, 13). Cytochrome c_2 shows an additional α helix beginning at residue *R*75 which is not found in cytochrome c.

Cytochrome c_2 exhibits amino acid sequence homology with horse heart cytochrome c at 43 positions, which are more or less equally distributed between surface and interior residues in the structures. Whereas 28 sequence positions of the 41 species of eucaryotic cytochrome used to tabulate Table II remain strictly invariant, cytochrome c_2 maintains strict sequence invariance at 15 of these positions and functional invariance at several additional sequence positions. The majority of the residues which are strictly invariant between c_2 and all species of eucaryotic cytochrome c form bonded or closest nonbonded interactions with the heme. These residues include: (*a*) Cys 17 which forms a thioether linkage to the heme (Cys 14 is replaced by Ala in two protozoan cytochrome c sequences (14–16)); (*b*) His 18 and Met (R91-*H*80) which form the fifth and sixth heme iron ligands, respectively; (*c*) Gly 29, Pro 30, and Leu 32 which form the right interior of the heme crevice (the carbonyl oxygen of Pro 30 accepts an H-bond from Nδ1 of His 18); (*d*) Tyr 48, Trp (*R*62-*H*59) which donate H-bonds to the rear heme propionate group, *R* Ser 49 and *H* Thr 49 form analogous H-bonds with the front heme propionic acid group; (*e*) Asn (*R*73-*H*70), Pro (*R*74-*H*71), Lys (*R*75-*H*72), Lys (*R*90-*H*79), and Phe (*R*93-*H*82) are located at the front left exterior of the heme crevice. Additional residues which are functionally and structurally homologous in the vicinity of the heme crevice include *R* Val 71-*H* Ile 68, *R* Ser 89-*H* Thr 78, and *R* Leu 95-*H* Ile 85.

Additional strict homology between c_2 and all eucaryotic cytochromes c is observed at glycine residues *R*2-*H*1, *R*7-*H*6, and 34. The first two residues are located in the NH_2 terminus α helix. Since glycine residues are usually considered to be helix-breaking residues, the invariance of these residues is somewhat anomalous, as noted previously (3). The invariance of Gly (*R*7-*H*6) can be rationalized on steric grounds, however, since it occurs at the interior interface of the terminal α helices in both molecules, a location which will not admit the substitution of even an alanyl side chain owing to the tight packing in this region. The invariant glycine 34 forms a type II_{10} (13) loop in cytochrome c_2, which is apparently not observed in the cytochrome c structures. The composite eucaryotic cytochrome c sequence (Table II, Column f) shows four additional in-

TABLE II

Comparison of sequential and structural homology of Rhodospirillum rubrum cytochrome c_2 and mitochondrial cytochrome c



a.) The solid line designates α-helical configuration; dashed line designates 3_{10}, Type I_{10} or II_{10} configuration (13) in R. rubrum cytochrome c_2.

b.) R. rubrum cytochrome c_2 sequence number.

c.) The solid bar indicates amino acid residues which are sequentially invariant with corresponding residues of horse heart cytochrome c. Open bar indicates c_2 residues which are functional and structural analogues of corresponding residues of horse heart cytochrome c.

d.) Amino acid sequence of R. rubrum cytochrome c_2 (sequence and homology with horse heart cytochrome c according to Dus et al. (11)).

e.) Amino acid sequence of horse heart cytochrome c (2).

f.) The solid bar indicates residues of the horse heart cytochrome c sequence which are found to be sequentially invariant in 38 species of eucaryotic cytochromes c of known sequence (2,5), as well as cytochromes c from the fungus Humicola lanuginosa (17), and two protozoans, Crithidia oncopelti (14), and Euglena gracilis (15,16). The open bar indicates residues of the horse heart cytochrome c sequence which are found to be conservatively replaced by residues in the remaining 40 eucaryotic cytochrome c sequences (i.e., the essential charged, hydrogen bonding, hydrophobic, or aromatic nature of the group is conserved).

g.) Horse heart cytochrome c sequence number.

h.) The bar designates α-helical configuration; dashed line designates 3_{10}, Type I_{10}, or II_{10} configuration. Some residues are blocked out by horizontal lines (i.e., Gly R2-H1), indicating sequential, structural, and functional homology between residues of the c_2 sequence and residues found to be invariant or conservatively substituted in all species of eucaryotic cytochrome c. The structural comments relevant to c_2 are derived from the structure determination of its oxidized form at 2 Å resolution (1). Preliminary examination of the ferrocytochrome c_2 structure by difference fourier methods indicates that no extensive conformational change has taken place on reduction, and it may be reasonably assumed that the commentary relevant to the oxidized form applies as well to the reduced structure. The tabulated data concerning eucaryotic cytochrome c is based on the published findings of Dickerson and coworkers (2-4). Assignment of the helical regions and hairpin loops (column h), however, are based on the ferrocytochrome c structure, since this appears somewhat better determined (2.45 vs. 2.8 Å resolution) than the oxidized form.

Abbreviations: H - horse; R - rubrum; A - Ala; C - Cys; D - Asp; E - Glu; F - Phe; G - Gly; H - His; I - Ile; K - Lys; L - Leu; M - Met; N - Asn; P - Pro; Q - Gln; R - Arg; S - Ser; T - Thr; V - Val; W - Trp; Y - Tyr.

variant glycine residues at positions *H*41, 45, 77, and 84 which are not observed in the c_2 sequence. The structural requirements subserved by *H* Gly 41 (*i.e.* the ability to form a tight bend) seem to be approximated by a pair of alanine residues (*R*40-41) in the c_2 structure. The strict invariance of *H* Gly 45 is not readily explainable on steric grounds, as has been commented upon by Dickerson (4). It is possible, however, that the folding pathway of the cytochrome *c* molecules necessitates the unusual degree of freedom in ϕ and ψ angles characteristic of glycine. Indeed, examination of the ferricytochrome *c* model shows a sharp chain reversal at *H* Pro 44-*H* Gly 45. In contrast, the sequentially homologous residue of the cytochrome c_2 sequence, *R* Asn 45, is H-bonded through its side chain amido group to *R* Asn 26 (see Reference 1, Fig. 3) which assists in rigidly constraining this region of the chain, as described previously (1). The tight loop formed by *H* Gly 77 has no analogue in cytochrome c_2 since this is the locus of an octapeptide insertion in the c_2 molecule. The invariant *H* Gly 84 likewise has no analogue in the c_2 molecule. Here, however, it is to be noted that the residues preceding *H* Gly 84 in the horse sequence, *H* Ile 81 and *H* Phe 82, undergo large conformational changes on oxidoreduction (see below) which presumably necessitate the presence of a glycine residue at *H*84 to accommodate the necessary ϕ and ψ angular rotations. Cytochrome c_2, in comparison, does not exhibit extensive conformational modification in this region on oxidoreduction.

The aromatic residues of cytochromes *c* and c_2 are strictly conserved only at positions Tyr 48, Trp (*R*62-*H*59), and Phe (*R*93-*H*82), as described above (see Table II). From Fig. 1, however, it can be seen that the majority of the aromatic residues, while not strictly homologous, pack in analogous ways in both molecules. (*a*) *R* Tyr 46, which is stacked parallel with the ring of Pro 30 and the hydroxyl of which donates an H-bond to the front heme propionate in c_2, is replaced by a similarly oriented *H* Phe 46 in horse cytochrome *c*. In tuna cytochrome *c* (and presumably, for several others in which the Phe to Tyr substitution is made) residue 46 is Tyr, and is H-bonded similarly to the corresponding c_2 residue, *R* Tyr 46. (*b*) Phe 36 occupies analogous positions in both molecules, although this residue is not invariant in eucaryotic cytochromes *c* in general. (*c*) The invariant residue *H* Phe 10, located on the upper right of the heme crevice, is deleted from the c_2 sequence, but appears to be

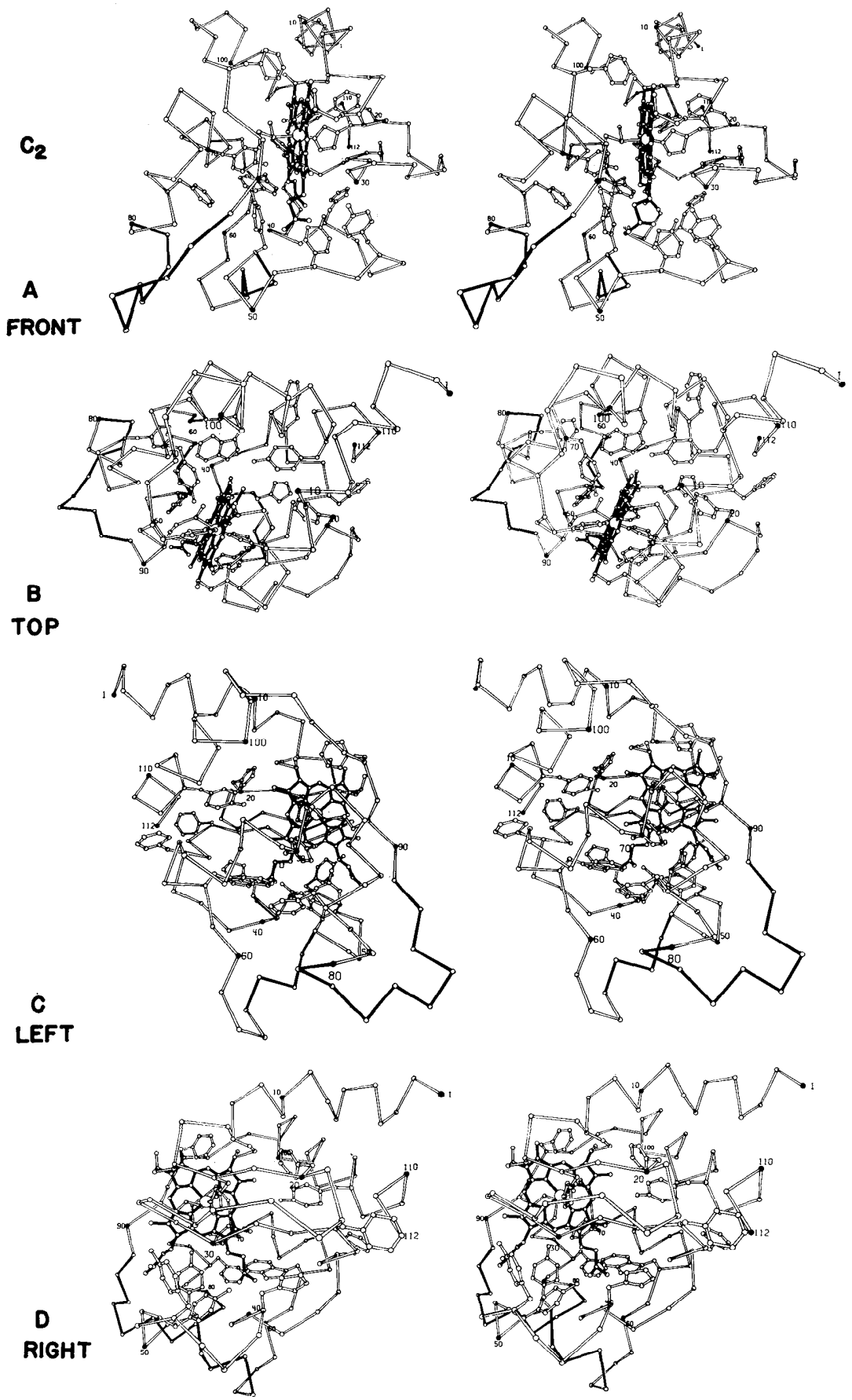


FIG. 1. Legend on p. 7705.

structurally approximated by *R* Phe 20 in the c_2 structure. (*d*) *H* Tyr 97 (Phe in some species), while sequentially homologous with *R* Tyr 107, is situated differently relative to the cytochrome *c* structure than is *R* Tyr 107 in the c_2 structure. The former is situated so that its hydroxyl group points out into, and is accessible to the solvent, whereas the latter is buried at the upper rear of the heme crevice. The difference in the relative orientations of these sequentially homologous residues results from the structural shift of the c_2 COOH-terminal α helix relative to its analogue in cytochrome *c*, as described above. (*e*) The residues *R* Phe 77 and *H* Tyr 74 occupy analogous positions on the left sides of the cytochrome *c* and c_2 molecules, although *R* Phe 77 is shielded from the solvent by the side chains of the c_2 octapeptide insertion at *R*80-*R*88. *H* Tyr 74 has been found to be substituted by Phe in two cytochrome *c* sequences (14, 17). (*f*) Tyrosine residues *R*70 and *H*67 occupy analogous positions adjacent to the left side of the heme in both molecules. The hydroxyl of *R* Tyr 70 forms an H-bond to the hydroxyl of *R* Tyr 52, which is in turn H-bonded to *R* Ser 89. *H* Tyr 67, by contrast, forms an H-bond directly with *H* Thr 78. It is notable that *H* Thr 78 (totally invariant in eucaryotic cytochrome *c* sequences) and *R* Ser 89 are sequential homologues, whereas the sequential homologue to *R* Tyr 52 is the totally invariant *H* Asn 52.

The significance of these variations of the aromatic residues to the physical properties and reactivity of cytochromes *c* and c_2 is discussed below.

Oxidation-Reduction Linked Conformation Changes—*R. rubrum* cytochrome c_2 crystallizes isomorphously in its oxidized and reduced forms and may be reversibly oxidized or reduced in the crystalline state (18, 19). Preliminary examination of a difference Fourier map between parent oxidized c_2 data and data obtained from dithiothreitol-reduced crystals indicates the absence of any large scale conformational change in cytochrome c_2 upon reduction.[1] This situation is to be contrasted with the case of mitochondrial cytochrome *c*, where it is found that the oxidized and reduced molecular forms crystallize in distinctly different conformations, consistent with numerous previous noncrystallographic observations (20–30) suggestive that oxidoreduction of cytochrome *c* is accompanied by a significant conformational change. Dickerson and co-workers (see References 4, 31–33) have described these conformational differences in detail.

In general, it is observed that upon reduction of ferricytochrome *c*, the molecule assumes a more compact configuration and that the heme appears to rotate slightly in its crevice as defined by the over-all configuration of the polypeptide chain. The most notable large scale change in conformation is the rotation of *H* Phe 82 from a solvent exposed position in the oxidized molecule (Figs. 1 and 2) to a position adjacent to the heme and restricting access to the heme iron in the reduced molecule. Concomitant with the inward rotation of *H* Phe 82 on reduction is the outward rotation of *H* Ile 81 from a position adjacent to the heme in the oxidized molecule, to a solvent accessible position in the reduced molecule. From Figs. 1 and 2 it can be seen that the homologue of *H* Phe 82, *R* Phe 93 is folded into the heme crevice in the ferricytochrome c_2 molecule, occupying a position analogous to that occupied by *H* Phe 82 in the ferrocytochrome *c* molecule.

As noted above, cytochrome c_2 lacks an exact homologue to the strictly invariant *H* Gly 84, which is proposed to act as a "hinge" to accommodate the extensive peptide bond rotations at *H* Ile 81 and *H* Phe 82 incurred during the oxidation-reduction-coupled conformational change in cytochrome *c*. Comparison of Fig. 1 of this communication with Fig. 10, Reference 4, would indeed suggest that cytochrome c_2 in both of its oxidation states most closely resembles the configuration of ferrocytochrome *c*, especially with regard to the packing of aromatic amino acid residues. These observations suggest that cytochrome c_2 is incapable of undergoing the extensive oxidation-reduction-linked conformational change exhibited by cytochrome *c*, and that it is constrained in a configuration which resembles that of ferrocytochrome *c*.

Surface Topography and Interactions with Physiological Oxidoreductants—One of the striking chemical characteristics of eucaryotic cytochrome *c* is its strongly basic isoelectric point which is a consequence of the large preponderance of positively charged groups on the surface of the molecule; *e.g.* horse heart cytochrome *c* has 22 positively charged groups (19 lysine residues, 2 arginine residues and the NH_2 terminus) *versus* 12 negatively charged groups (3 aspartic acid, 8 glutamic acid, and the COOH terminus) on its surface. A variety of studies have indicated the importance of the positively charged groups, specifically the lysines, in the interactions of cytochrome *c* with physiological oxidoreductants, namely:

1. Harbury and co-workers (34) found that trifluoroacetylation of all of the lysine residues in horse heart cytochrome *c* rendered the product inactive in the succinate oxidase system, whereas a totally guanidinated derivative was found to react comparably to the unmodified protein in the same system (35, 36).

2. White and Elliot (37) have studied the reaction of horse heart cytochrome *c* with gladiolic acid, an antifungal metabolite produced by *Penicillium gladioli*, and related orthodialdehydes which react selectively with lysine residues. They observed that a product into which 3 moles of gladiolic acid per mole of cytochrome *c* had been incorporated lost 85% of its activity in the beef heart cytochrome oxidase system, and 100% of its activity in pig-heart NADH-cytochrome *c* reductase system.

3. Wada and Okunuki (38) observed that a monosubstituted derivative of horse heart cytochrome *c* trinitrophenylated at *H* Lys 13 was 50% as active as the native protein in the bovine cytochrome oxidase system.

4. Smith and co-workers (39, 40) have demonstrated that polylysine and other polycations are potent inhibitors of the reaction of cytochrome *c* with both cytochrome oxidase and NADH cytochrome *c* reductase (41).

The foregoing observations imply that the interactions of cytochrome *c* with its physiological oxidoreductants are facilitated by a charge interaction between the positively charged lysine residues of cytochrome *c* and complementarily charged sites on its oxidase and reductase (39, 41).

R. rubrum cytochrome c_2, while sharing an over-all configuration that is similar to that of cytochrome *c*, exhibits an isoelectric point of 6.3 (42), having 18 positively charged groups (17 lysines and the NH_2 terminus) and 16 negatively charged groups (6 aspartic acid, 9 glutamic acid, and the COOH terminus) on its

[1] F. R. Salemme, S. T. Freer, R. A. Alden, and J. Kraut, manuscript in preparation.

FIG. 1. Stereo drawings of *R. rubrum* ferricytochrome c_2 (1) showing α-carbon positions, heme ligands, and aromatic residues from four aspects: *A*, *front*; *B*, *top*; *C*, *left side*; *D*, *right side*. The heme and the principle sequence and structural insertions into the eucaryotic cytochrome *c* sequence are shown in back (see Table II).

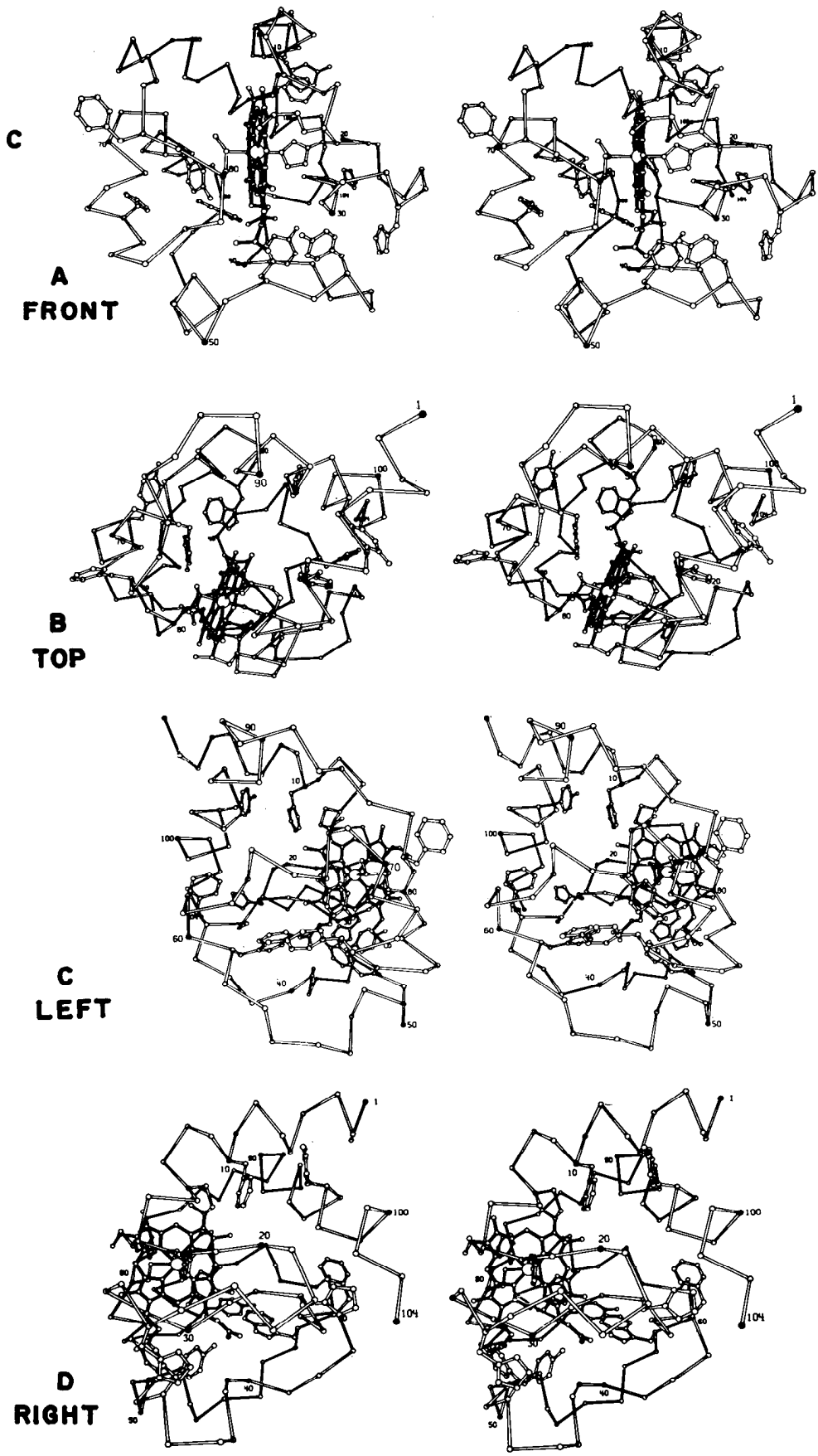


FIG. 2. Stereo drawings of eucaryotic mitochondrial ferricytochrome *c* (horse, tuna) according to Dickerson *et al.* (3), showing α-carbon positions, heme ligands, and aromatic residues from four aspects equivalent to those in Fig. 1: *A*, *front*; *B*, *top*; *C*, *left side*; *D*, *right side*.

surface. Nevertheless, cytochrome c_2 will react with components of the mitochondrial electron transport chain. This observation, originally reported by Elsden *et al.* (6), has recently been extended (43) with the following results. (*a*) *R. rubrum* cytochrome c_2 exhibits approximately 60% of native horse heart cytochrome *c* activity in the solubilized DPNH-cytochrome reductase system. The reduction of cytochromes *c* and c_2 was totally inhibited by antimycin A (an agent which blocks reduction of the cytochrome c_1 which is the component of the reductase serving as the electron donor to cytochrome *c*), and approximately 98 and 85% inhibited, relative to their respective rates, in the presence of 5 μM polylysine (mol wt 15,000). Michaelis constants for cytochromes *c* and c_2 were 13.3 μM and 23.8 μM, respectively. (*b*) Cytochrome c_2 was observed to react with cytochrome oxidase with approximately 5% of the activity observed with cytochrome *c* at a similar concentration (11 μM). Inhibition of the cytochrome *c* oxidation was complete in the presence of 5 μM polylysine, whereas cytochrome c_2 oxidation was approximately 75% inhibited under the same conditions. Increasing the cytochrome c_2 concentration to 100 μM gave an activity which was approximately 25% of that observed with 11 μM cytochrome *c*, and was 90% inhibited by 5 μM polylysine.

Several inferences concerning the relative reactivities of cytochromes c_2 and *c* follow from these results. (*a*) The sensitivity of the reduction of both cytochromes *c* and c_2 to antimycin A, as well as the comparable Michaelis constants of cytochromes *c* and c_2 suggest essential similarity in the binding sites of these cytochromes to the reductase. The potent inhibition of the reduction by polylysine of both cytochromes *c* and c_2 further suggests that the binding of both cytochromes *c* and c_2 is mediated predominantly by a charge interaction involving the lysine residues of the cytochromes and anionic groups of the reductase. (*b*) Cytochrome c_2 does not react proportionately as well with cytochrome oxidase, relative to cytochrome *c*, as it does with the reductase. Nevertheless, the effectiveness of polylysine in inhibiting the oxidation of both cytochromes *c* and c_2 again suggests that the interaction between the acidic oxidase and cytochromes *c* is predominantly due to complementary charge interaction between these molecules.

The question which is immediately raised by these observations is whether or not the proposed cationic binding sites to the oxidase and reductase which are presumed to be involved in both cytochromes *c* and c_2, constitute common or different binding sites on the surface of the cytochrome *c* and c_2 molecules. Fig. 3 shows the comparative surface topography of the cytochrome *c* and c_2 molecules from three aspects. It is readily seen from Fig. 3, *A* and *B* that the cytochrome *c* and c_2 molecules share in common an essentially uninterrupted distribution of lysine residues around the perimeter of their heme crevices at the front of the molecule. This feature is manifest particularly well in the case of cytochrome c_2, where 11 of the total 17 lysine residues of the molecule are located at the front of the molecule, resulting in a pronounced asymmetry in the charge distribution on the surface of the nearly electrically neutral molecule. Of the 11 lysine residues distributed around the heme crevice in cytochrome c_2, 6 (Lys *R*9-*H*8, *R*12-*H*13, *R*27-*H*27, *R*75-*H*72, *R*90-*H*79, *R*97-*H*87) are sequential and structural homologues between cytochrome c_2 and horse heart cytochrome *c*. In addition, 4 of the 7 basic charge-invariant residues common to all eucaryotic mitochondrial cytochromes *c*, residues *H*13, *H*72, *H*79, and *H*87, are located in the positive charge belt around the heme and are sequentially and structurally homologous with residues in c_2. Of the remaining cytochrome *c* invariant basic residues, *H* Lys 73 appears to have a structural analogue in *R* Lys 88, whereas c_2 does not have structural or sequential analogues to *H* Arg 38 and *H* Arg 91, which may play some role in the oxidation-state linked anion binding characteristics of eucaryotic cytochromes *c* (44–46).

Fig. 3, *B* and *C* show the comparative topography of the left sides of the molecules. Although there is reasonable correspondence in the location of the lysine residues, the striking correspondence seen for the front of the molecule, especially with respect to strictly invariant charged residues is not found. Dickerson and co-workers (4) have proposed that the left side of the cytochrome *c* molecule serves as the binding site to cytochrome reductase, and that reduction takes place by means of a free radical pathway (47) involving the surface residue *H* Tyr 74, and the internal residues *H* Tyr 67 and *H* Trp 59. This proposal is called into question by several observations. Whereas the high reactivity of cytochrome c_2 with cytochrome reductase would suggest similarity in the reactive sites of cytochrome *c* and c_2, the left side of cytochrome c_2 is most distinctly different from that of cytochrome *c*, both by virtue of its surface charge topography and the fact that the left side of the c_2 molecule is the locus of an octapeptide insertion into the cytochrome *c* sequence. In addition cytochrome c_2 has *R* Phe 77 in the sequentially homologous position to *H* Tyr 74. Although not readily apparent from the α-carbon diagrams of Fig. 1, the ring of *R* Phe 77 (as remarked above) is shielded from the external environment by the side chains of the octapeptide insertion at *R*80-88. Indeed, the reduction of *R* Phe 77 implicit in a free radical reduction mechanism via this residue would seem an extremely unfavorable process energetically.

The attractiveness of the left-side binding free radical scheme previously rested on the invariance of the aromatic residues proposed to be involved in the pathway (*H* Tyr 74, *H* Tyr 67, *H* Trp 59), and on the invariance of the section of the cytochrome *c* sequence from *H*70 to *H*80, which forms a loop (see Fig. 2) roughly covering the lower left front octant of the molecule. Recently, however, two cytochrome sequences from the thermophilic fungus *Humicola lanoginosa* (17) and the protozoan *Crithidia oncopelti* (14) have been determined, both of which have a phenylalanine substituted at position *H*74. In addition, it has been recently found that the previously invariant *H* Tyr 67 is replaced by Phe in *Euglena gracilis* cytochrome *c*-558, which on the basis of its extensive homology with mitochondrial cytochromes *c* (15, 16) and its association with the algal oxidase system, must be included in this class. Indeed it has been shown that *Euglena c*-558 exhibits good reactivity in the solubilized mitochondrial oxidase and reductase systems (200% and 68% of horse heart cytochrome *c* activity, respectively (43)).

Inclusion of the strictly invariant *H* Trp 59 in the free radical reduction scheme was suggested by the *H* Trp 59 oxidation experiments of Myer (48–50) who found that reduction of the modified horse heart cytochrome *c* by NADH cytochrome *c* reductase was inversely proportional to the amount of tryptophane destroyed, whereas its oxidation in the succinate oxidase system was less affected. Although this result is suggestive that *H* Trp 59 is directly involved in the reduction mechanism of cytochrome *c*, it should be noted that this residue, by virtue of its hydrogen bonding to the buried rear heme propionic acid group, plays an important role in maintaining the proper configuration of the heme crevice in cytochrome *c* (1, 3, 51). Thus, Aviram and Schejter (52) noted that formyl-*H*- Trp 59 ferricytochrome *c* (a substitution at the tryptophane indole nitrogen abolishing its ability to form a hydrogen bond with the heme

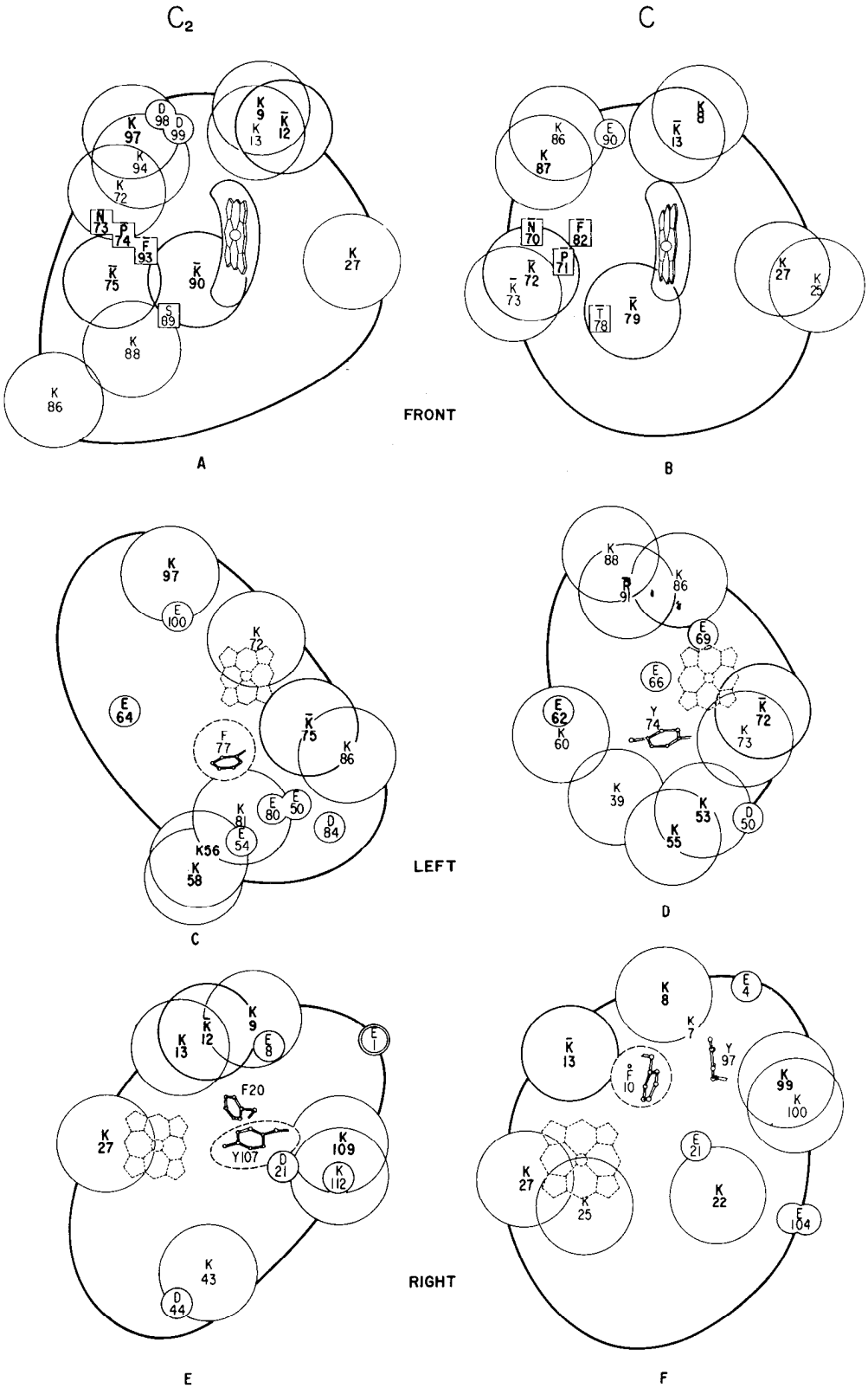


FIG. 3. Schematic diagrams of the surface features of the cytochrome c_2 and horse cytochrome c molecules. Residues in *bold face* are structurally and sequentially invariant between horse cytochrome c and *R. rubrum* cytochrome c_3. Residues in *bold face* with *bar* are structurally homologous between cytochrome c_2 and horse heart c and are sequentially strictly invariant among the 41 species of eucaryotic cytochrome c included in Table II. Residues having the *bold bar* only are sequentially strictly invariant among 41 species of eucaryotic cytochrome c. The *circles* about the α-carbon positions of the lysine residues give the approximate radius of flexibility for lysine side chains. (Amino acid letter code is given in caption to Table II.) *A*, c_2 *front*; *B*, horse cytochrome c *front* (*H* Lys 13 is Arg in some species, *H* Lys 72 is trimethyllysine in some species); *C*, c_2 *left side*, showing location of *R* Phe 77 which is shielded from external solvent; *D*, horse cytochrome c *left side* showing position of *H* Tyr 74; *E*, c_2 *right side* showing relative orientations of the partially exposed ring of *R* Phe 20 and the buried ring of *R* Tyr 107; *F*, horse cytochrome c *right side* showing the relative positions of *H* Phe 10 and *H* Tyr 97.

propionate), lacked the 695 nm absorption band characteristic of native ferricytochromes *c* (53–55), which is identified with methionine sulfur ligation to the heme iron (56). Further, from a variety of studies (53, 57–60) it is apparent that the *H* Met 80 sulfur-reduced heme iron bond is some 100 to 1000 times stronger than the bond formed between the methionine sulfur and the oxidized heme iron. These observations imply that the hydrogen bond formed between the rear heme propionate and *H* Trp 59 is relatively more important in maintaining the integrity of the heme methionine ligand in the oxidized form of the molecule, because the iron-sulfur interaction is much weaker, than in the reduced molecule. This would suggest that the observed differential in the oxidase and reductase activities of *H* Trp 59-modified-cytochrome *c* arose because the species reacting with the oxidase (reduced modified-cytochrome *c*) retained the essential integrity of the *H* Met 80 heme ligand, whereas the oxidized species had lost the sixth ligand and therefore was incapable of reacting with the reductase.

In any case it can be seen from Table II and Fig. 3 that those residues of the cytochrome c_2 sequence which are identical with the totally invariant residues of mitochondrial cytochrome *c* in the region *R*74-*H*70 to *R*93-*H*82 all are located at the left front surface of the c_2 molecule (Fig. 3, *A* and *B*), with the exception of Met (*R*91-*H*80) which is somewhat buried by virtue of its ligation to the heme iron. These residues include Asn (*R*73-*H*70), Pro (*R*74-*H*71), Lys (*R*75-*H*72), Lys (*R*90-*H*79), and Phe (*R*93-*H*82). The invariant *H* Thr 78 appears to be functionally conserved in the c_2 structure by *R* Ser 89 (see Fig. 3 and Table II).

Fig. 3, *C* and *D* show the topographic charge distribution on the right sides of the cytochrome *c* and c_2 moleulces. Both molecules show a ringlike distribution of lysine residues, which is to some extent broken up by surface acidic residues, around a central hydrophobic patch. This feature of the cytochromc *c* molecule has been described by Dickerson and co-workers (2–4) as the "right channel" since the distance between the aromatic rings of *H* Phe 10 and *H* Tyr 97 would permit intercalation of an additional aromatic ring in the oxidized form of the molecule. In ferrocytochrome *c*, access to this region becomes restricted due to a general upward displacement of the chain from *H*19 to *H*27 which forms a loop in the right side of the molecule (Fig. 2). Although cytochrome c_2 has two aromatic residues *R* Phe 20 and *R* Tyr 107 positioned similarly to those forming the right channel in cytochrome *c*, the disposition of these residues is somewhat different from that observed in cytochrome *c* as described above.

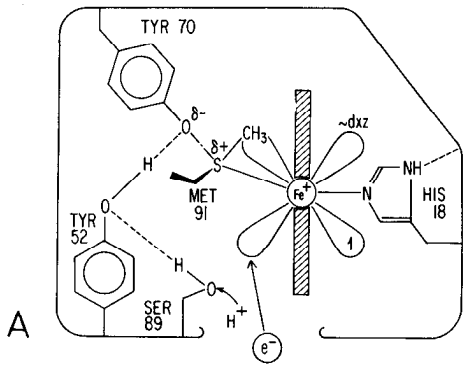
It has been suggested by Dickerson *et al.* (2–4) that the right side of the cytochrome *c* molecule serves as the binding site to the oxidase, and indeed the fact that the introduction of the bulky trinitrophenyl group onto *H* Lys 13 abolishes 50% of the oxidase activity (41) may suggest that this is true. (Here it should be noted that *H* Lys 13, by virtue of its location at the upper right of the heme crevice, may form interactions either at the front or right side of the molecule due to the length and flexibility of its side chain; see Fig. 3, *B* and *F*.) In this case the poor reactivity of cytochrome c_2 with the oxidase would be rationalized in terms of the different configurations of the aromatic residues on the right sides of the two cytochrome molecules. There remains the difficulty, however, as to how oxidation would take place via a right side pathway, since such a mechanism would again appear to invoke the existence of an energetically unfavorable aromatic free-radical intermediate.

Alternatively, if it is assumed that *H* Lys 13 when trinitrophenylated, assumes a configuration similar to that observed in the crystalline structure of the ferri- or ferrocytochrome *c* molecules (in which *H* Lys 13 lies on the front of the molecule in the immediate vicinity of the heme crevice), the lowered activity of the trinitrophenylated derivative can be envisioned as a result of hindered access to the exposed edge of the heme at the front of the molecule by the oxidase. If the front side is the site of the oxidase interaction with cytochrome *c*, it is necessary to account for the low reactivity of cytochrome c_2 which exhibits a striking resemblance to cytochrome *c* in this region. This can be done by postulating, as in the case of enzymes which interact with small substrates (61), that the oxidase and reductase bind a conformational species of cytochrome *c* which resembles an intermediate structure between the oxidized and reduced conformations (*i.e.* a transition state). The low reactivity of cytochrome c_2 with the oxidase may now be rationalized by noting that c_2 is incapable of undergoing a oxidation-reduction-linked conformational change, but conformationally resembles reduced cytochrome *c* in both of its oxidation states. The foregoing concept is central to the hypothesis presented in this paper, and will be discussed in greater detail in the next section.

As a result of these structural observations, we feel that the similarities in the behavior between cytochromes *c* and c_2 are best explained by the assumption that both cytochromes *c* and c_2 interact with their physiological oxidoreductases at the exposed edge of the heme at the front of the molecule. Recently, however, Smith *et al.* (62) have isolated an antibody Fab fragment which appears to inhibit selectively the reaction of cytochrome *c* with its oxidase, while leaving its reductase activity intact. Since a bound Fab fragment would be expected on the basis of its size effectively to mask at least an octant of the cytochrome *c* surface, this result would appear to suggest that the sites of interaction of cytochrome *c* with its oxidase and reductase are spatially separated on the surface of the molecule. It is possible, however, since cytochrome *c* undergoes such an extensive oxidation-reduction-linked conformational change, that the inhibition of the oxidase activity is not due to the antibody being bound at the site of interaction with the oxidase, but at some remote site which structurally prohibits transition to a conformation capable of oxidation by the oxidase, in analogy with the situation proposed to account for the low reactivity of cytochrome c_2 with the oxidase. Indeed the difference in the reactivity of *H* Tyr 74 (located on the left side of the molecule) toward iodination in ferri and ferrocytochrome *c*,[2] along with the observed changes in the accessibility of *H* Tyr 97 and *H* Phe 10 (located on the right side) in the oxidized and reduced molecule, suggest plausible sites for antibody interaction remote from the front of the molecule which, when constrained by antibody binding, could account for the selective loss of activity.

Alternative Proposal for Mechanism of Oxidoreduction of Cytochrome c—A physiological mechanism for the oxidoreduction of cytochrome c_2 has been recently proposed in which electron addition to and withdrawal from the heme takes place by an essentially reversible reaction involving direct interaction of the electron donating or withdrawing group with the exposed edge of the heme, accompanied by the perturbation of a hydrogen bond network postulated to stabilize the heme in its oxidized state (1). The proposed mechanism (Fig. 4) for c_2 oxidoreduction appears to be supported by a variety of structural and physicochemical properties of the molecule. These properties are compared and contrasted with the corresponding properties

[2] M. Morrison, private communication.



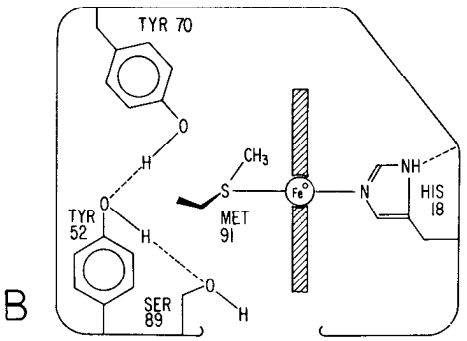
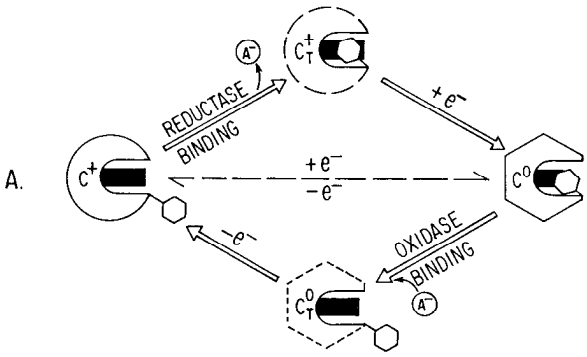


FIG. 4. The proposed mechanism of *R. rubrum* cytochrome c_2 oxidoreduction. *Part A* shows the region of the ferriheme, whose unpaired spin is localized in the *xz* plane, where the *z* axis is horizontal and *x* axis vertical in the plane of the paper (Fig. 7*A*). Stabilization of the ferriheme iron charge is achieved by an ionic interaction between the hydroxyl oxygen of *R* Tyr 70, bearing a partial negative charge, and the sixth ligand *R* Met 91 sulfur atom, bearing a partial positive charge delocalized from the iron. Reduction is facilitated by protonation of *R* Ser 89, tending to destabilize the oxidized state, concomitant with electron donation to the heme. *Part B* shows the configuration of the reduced heme, where the charge interaction between *R* Tyr 70 and the sulfur *R* Met 91 has been abolished due to the loss of the heme iron charge. Oxidation is postulated to take place essentially by the reverse of the reduction process.



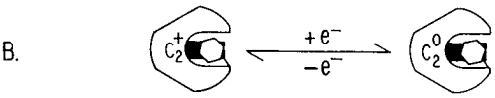


FIG. 5. *A*, schematic for the physiological oxidoreduction of eucaryotic cytochrome *c*. The oxidized molecule (C^+) is destabilized on binding to the reductase by displacement of a bound anion (A^-). The destabilized oxidized state ($C_T{}^+$) is enzymatically reduced by the reductase, giving rise to the reduced molecular conformation C^0. Upon binding to the oxidase, the reduced form is destabilized by the opening of the heme crevice ($C_T{}^0$), facilitating enzymatic oxidation by the oxidase, resulting in the oxidized conformation (C^+). The *dotted arrow* between the oxidized (C^+) and reduced conformation (C^0) indicates the pathway for nonphysiological oxidoreduction of cytochrome *c*, which does not take place via the intermediates of the physiological mechanism. *B*, schematic showing the oxidoreduction of *R. rubrum* cytochrome c_2, which does not undergo an extensive oxidation-reduction-linked conformational change. Structural similarity between the oxidized form of c_2 and $C_T{}^+$ above accounts for the efficiency with which oxidized c_2 is reduced by the mitochondrial reductase. The inability of c_2 to attain a conformation resembling $C_T{}^0$ accounts for its poor reactivity with the mitochondrial oxidase.

of mitochondrial cytochrome *c* in the following section. The extensive and predominant structural similarity of the cytochrome c_2 and *c* molecules, both with regard to the residues which are nearest neighbors to the heme and its ligands, and with regard to the surface residues distributed about the perimeter of the crevice, suggests that mitochondrial cytochrome *c* may undergo physiological oxidoreduction by a mechanism that is in principle similar to that proposed for cytochrome c_2. A simplified schematic of the proposed cytochrome *c* oxidoreduction process is shown in Fig. 5 to assist the reader in following the detailed structural and mechanistic arguments presented below.

Fig. 6*A* shows a schematic of the heme region of ferricytochrome *c*. Stabilization of a positive charge on the ferriheme iron is achieved by the binding of an anion on the upper left of the heme crevice, which is in turn stabilized by the proximity of the ammonium group of *H* Lys 13. On front side approach of the reductase, *H* Phe 82 is pushed into the heme crevice, displacing the stabilizing anion and *H* Lys 13. This causes some positive charge to be delocalized to the easily polarizable sulfur atom of *H* Met 80, which is in turn transiently stabilized by a partial ionic interaction with the hydroxyl oxygen of *H* Tyr 67. The amide group of *H* Asn 52 may move from its position in the oxidized molecule, where it is hydrogen bonded to the front heme propionic acid group, and assist in stabilizing a partial negative charge on the hydroxyl oxygen of *H* Tyr 67 (Fig. 6*B*). Reduction takes place by electron donation to the heme from a closely juxtaposed group of the reductase. This process may be aided by the action of a suitably positioned group on the reductase capable of protonating *H* Thr 78 (in analogy with the scheme shown for c_2 in Fig. 4*A*), which would in turn decrease the extent of ionic stabilization between the hydroxyl of *H* Tyr 67 and the sulfur of *H* Met 80, and increase the positive charge localization on the heme iron, facilitating its reduction. Upon reduction of the heme iron, the partial ionic interaction between the sulfur of *H* Met 80 and the hydroxyl oxygen of *H* Tyr 67 is abolished and the sulfur iron coordinate bond becomes some 100 to 1000 times stronger than in the oxidized molecule (Fig. 6*C*).

It is the great increase in strength of the *H* Met 80 sulfur-heme iron coordinate bond which is the probable prime mover in the oxidation-reduction-coupled conformational change of cytochrome *c*, because any local strains induced by this interaction would be propagated throughout the molecule by virtue of the extensive bonded and nonbonded interactions of the heme and its ligands with the remainder of the polypeptide chain. Part of the conformational change in the lower left octant of the molecule results from a small shift of the polypeptide chain in the region of *H* Asn 52, which is proposed to transiently stabilize the partial negative charge on the hydroxyl of *H* Tyr 67 in the

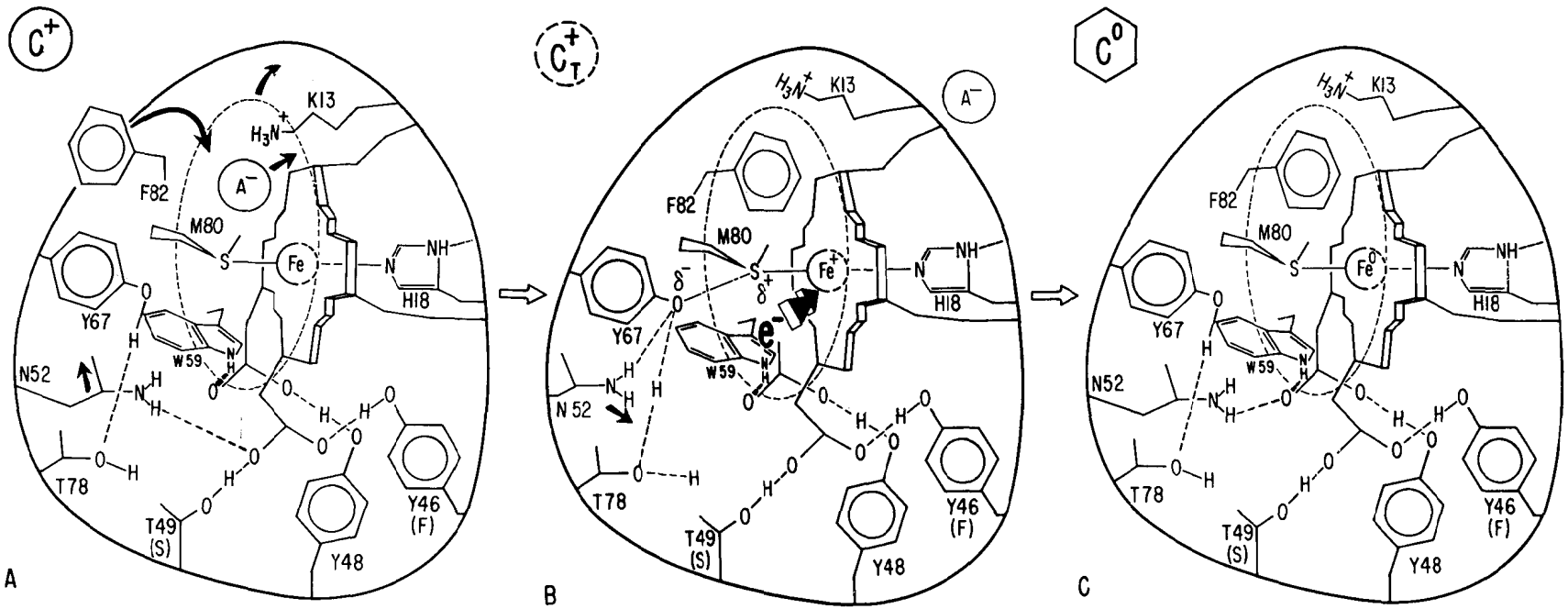


FIG. 6. Proposed mechanism for the physiological oxidoreduction of eucaryotic cytochrome *c*. *A*, ferricytochrome *c* heme region showing groups which form bonded heme interactions. Stabilization of the ferriheme iron charge is achieved by a bound anion, which is in turn stabilized by a charge interaction with the ε-amino group of *H* Lys 13. *B*, on reductase binding, the ring of *H* Phe 82 is pushed into the heme crevice, displacing the bound anion. Heme iron charge stabilization is transiently achieved by a partial ionic interaction between the hydroxyl oxygen of *H* Tyr 67 and the sulfur atom of *H* Met 80 bearing some positive charge delocalized from the ferriheme iron. Reduction takes place by direct electron transfer from a closely juxtaposed group of the reductase, and may be facilitated by perturbation of the hydrogen bond system formed by *H* Thr 78 and *H* Tyr 67. *C*, on reduction the partial ionic interaction between *H* Met 80 and *H* Tyr 67 is abolished, concomitant with a great increase in the *H* Met 80 sulfur-heme iron bond strength, which induces the over-all transition to the reduced molecular conformation. Reoxidation takes place essentially by the reverse of the reduction mechanism, *i.e.* the reduced molecule binds to the oxidase which abstracts the ring of *H* Phe 82 from, or inserts an anionic group into the heme crevice, or both, facilitating reoxidation (see text for details).

transition state. The loss of the internal ionic interactions on heme reduction allows this residue to move to a position in the reduced conformation where it is H-bonded to the rear heme propionate group.

Reoxidation of the reduced molecule is proposed to take place by essentially the reverse of the above mechanism, whereby oxidation is facilitated either by the abstraction of the ring of *H* Phe 82 and insertion of some negatively charged group of the reductase, or by the interaction of some proton withdrawing group with *H* Thr 78, or a combination of these factors. In either case, reoxidation is facilitated by a change in the charge environment at the heme iron tending to stabilize the oxidized state of the molecule.

Comparison of Structural and Physicochemical Bases for Proposed Oxidoreduction Mechanisms of Cytochrome c and c_2—The configurations of ferri and ferrocytochrome *c* as shown in Fig. 6, *A* and *C* are those reported by Dickerson and co-workers (3, 4). Dickerson has proposed that an anion is bound in a position adjacent to the iron in the oxidized molecule which serves to stabilize the ferriheme charge (63). This proposal is based upon the fact that a large positive peak is observed in the electron density map of the oxidized molecule in the position adjacent to the heme occupied by the ring of *H* Phe 82 in the reduced molecule (4). It is further supported by the observation that ferricytochrome *c* crystallizes only in the presence of chloride ion. Stabilization of a positive charge on the ferriheme iron of cytochrome c_2 by anion binding in this region is not possible, because *R* Phe 93 (the homologue of *H* Phe 82) is situated adjacent to the heme in both oxidation states of the molecule (Figs. 1 and 7).

Instead, stabilization of the c_2 ferriheme is achieved by a charge interaction formed between the hydroxyl oxygen of *R* Tyr 70 and the *R* Met 91 sulfur atom bearing a partial positive charge delocalized from the iron, as is evidenced by the observation that the sulfur atom of *R* Met 91 appears to be displaced off axis from the Fe-Nϵ2 His 18 bond toward the hydroxyl oxygen of *R* Tyr 70, from which it is ~3 A distant (Figs. 4 and 7). In contrast, the calculated distance between the hydroxyl oxygen

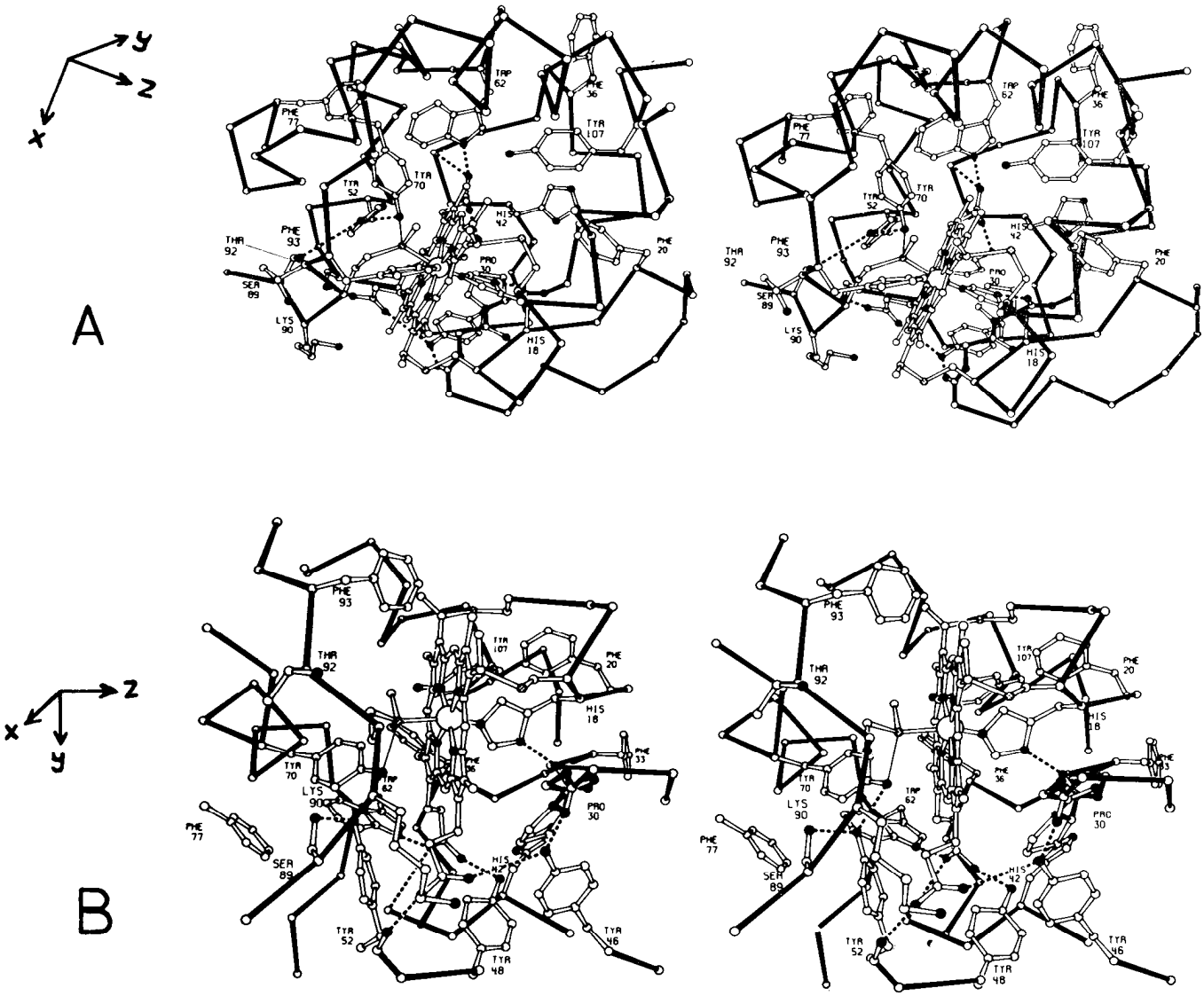


FIG. 7. Stereo drawings of the heme region of ferricytochrome c_2 showing the interactions of the heme and its ligands. *A*, *top view*, corresponding to Fig. 4. *B*, *front view*, in same relative orientation as Fig. 6. Note the similarity between the interactions formed by the heme and its ligands in c_2, and that of the proposed intermediate of cytochrome *c* shown in Fig. 6*B*. The orientation of the d_{xz} heme iron orbital in Figs. 6 and 7*B* is such that the *z* axis lies horizontal in, and the *x* axis perpendicular to the plane of the paper.

of *H* Tyr 67 and the sulfur atom of *H* Met 80 in ferricytochrome *c* is ~4.4 A, and indeed distortion of the sulfur-iron bond from the expected axial position is not observed in ferricytochrome *c* structure at its current resolution. The rather poorer effectiveness of the internal ionic stabilization of the ferriheme iron of c_2 *versus* the stabilization afforded by the more open crevice and bound anion stabilization seen in ferricytochrome *c* correlates well with the higher oxidation-reduction potential of the former (32, 64, 65).

Upon the approach of cytochrome *c* to the reductase, the ring of *H* Phe 82 is pushed into the heme crevice, which causes the bound anion to be displaced, concomitant with an upward shift of *H* Lys 13. (The fact that the inward rotation of *H* Phe 82 is a necessary result of the front-side approach to the reductase can be seen from Fig. 2 where the protuberance of *H* Phe 82 in the ferricytochrome *c* molecule is clearly shown.) Once the stabilizing anion is displaced, ferriheme stabilization is achieved by partial ionic interaction between the sulfur of *H* Met 80 and the hydroxyl oxygen of *H* Tyr 67, the partial negative charge induced on the *H* Tyr 67 hydroxyl oxygen being additionally stabilized by an interaction with the amide group of *H* Asn 52. Participation of *H* Asn 52 in the stabilizing network is suggested principally by the structural observations that the side chain of *H* Asn 52 need only rotate about its C_α-C_β bond in order for its amide group to be situated proximal to the hydroxyl oxygen of *H* Tyr 67 in the oxidized molecule, and that this position is structurally intermediate between the locations this residue occupies in the oxidized and reduced conformations of the cytochrome *c* molecule. Participation of the eucaryotically invariant *H* Asn 52 in the transition state stabilization is further suggested by the striking similarity seen between the proposed intermediate conformation of cytochrome *c* (Fig. 6*B*) and the oxidized conformation of cytochrome c_2 (Figs. 4*A* and 7), where the groups which form hydrogen bonds with the rear heme propionate (*H* Tyr 48-*R* Tyr 48, *H* Trp 59-*R* Trp 62) are strictly conserved, and the role of *H* Asn 52 is approximated by the statically positioned *R* Tyr 52. Indeed, given the facts that cytochrome c_2 does not undergo any extensive oxidation-reduction coupled conformation change and that the groups involved in the facilitation of heme oxidoreduction are statically positioned (*i.e.* *R* Tyr 70, *R* Tyr 52, and *R* Ser 89 do not change conformation on reduction of the molecule), it would appear that the conformation of cytochrome c_2 represents an analogue of the oxidation-reduction transition state (C_T^+ in Fig. 5*A*) of cytochrome *c*.

Cytochrome *c* heme reduction is proposed to take place, subsequent to attainment of the transition state, by a process of direct electron addition to the heme, facilitated by perturbation of the *H* Thr 78-*H* Tyr 67 hydrogen bond system which is of predominant importance in stabilizing the buried positive charge on the ferriheme iron in the transition state.

The proposal that heme reduction takes place by direct electron addition to the heme is suggested by several studies concerning the localization of the unpaired spin density in the ferricytochrome *c* and c_2 molecules. Specifically, the high resolution NMR spectra[3] of both cytochromes *c* and c_2 show essential similarity in the locations, intensities, and heme contact shifts of resonances which have been assigned to protons of the axial heme ligands and heme methyl groups (66, 67). From these observations, it can be argued in simple terms (see Reference 1, also 68, 69), that it is the d_{xz} orbital which is partially unoccupied in both ferricytochromes *c* and c_2, where the coordinate system is defined with the Fe-N_2 pyrrole bond lying along the x axis, the Fe-N_1 pyrrole bond lying along the y axis, and the Fe-Nϵ2 His 18 bond lying along the z axis. As was noted previously (1), one lobe (Figs. 4, 6, and 7) of this orbital lies along the shortest and most accessible route from the protein exterior to the heme iron. Further, since reduced cytochromes *c* and c_2 exhibit a net spin of zero, it is implied that the reducing electron goes into this orbital on heme reduction. Experiments upon the reduction of ferricytochrome *c* by hydrated electrons (70–74) also suggest that reduction takes place by a direct route, despite the fact that the reduction potential of the solvated electron is equivalent to that of an electron delivered from an electrode at a potential of some −2.8 volts, which should be capable of producing a radical anion of virtually any aromatic group of the protein (75, 76).

The principal chemical evidence which suggests the importance of the *R* Ser 89-*R* Tyr 52-*R* Tyr 70 hydrogen bond system in the stabilization of ferricytochrome c_2 is its E_m *versus* pH behavior. The observed midpoint potential of c_2 falls from +370 mv at pH 5 to 300 mv at pH 8, after which it remains constant to pH 10 (77). This behavior is consistent with the oxidation-reduction linked titration of a group having an apparent pK of ~7.8 according to the equation $c_2(\text{ox}) + H^+ + e^- \rightleftarrows c_2$ (red), where the ionizing species is tentatively identified with one or more of the groups involved in the c_2 ferriheme stabilizing system. The E_m *versus* pH behavior of cytochrome *c* is markedly different: the potential remains constant at ~+250 mv from pH 2 to 7.8, after which it begins to drop such that the observed potential at pH 10 is ~+120 mv (78). This property of cytochrome *c* has been the subject of extensive investigations (79–83), and it would appear that the observed pH *versus* E_m behavior is well explained by the following scheme:

$$\begin{array}{ccc} c(\text{ox}) + e^- & \rightleftarrows & c(\text{red}) \\ \Updownarrow & \longrightarrow & H^+ \\ c^*(\text{ox}) & & \end{array}$$

where *c*(ox) is the neutral pH native form of cytochrome *c* capable of rapid reduction by the reducing species, and *c**(ox) is a conformational isomer of cytochrome *c* predominating at alkaline pH which is not easily reduced by small ion reductants of moderate reduction potential. The striking correlations between pH dependence of the reducibility of cytochrome *c* by hydrated electrons (74) and ascorbate (55, 82), the magnitude of the 695-nm absorption band identified with the integrity of the heme-*H* Met 80 sulfur ligand (55, 74, 82), the magnetic susceptibility (54), and the magnitude of the NMR contact shifted methyl resonance attributed to *H* Met 80 in ferricytochrome *c* (60, see also 84), all suggest that the alkaline form of cytochrome *c* has either an alternative sixth ligand (*H* Lys 79 being the most likely candidate (85)) or at least that the nature of the *H* Met 80 sulfur-heme iron bond has changed. Gupta and Koenig (60) have postulated that deprotonation of *H* Lys 79 leads to the formation of the alkaline conformer (*c**(ox)) of cytochrome *c*. However, their observations appear equally consistent with the proposal that *H* Tyr 67 is the responsible ionizing species. The latter is suggested by the close correlation between the pH *versus* the intensity of the 695-nm band and hydrated-electron reducibility of ferricytochrome *c* which has been nitrated at tyrosine 67 (86–88). Thus, it was found that the intensity of the 695-nm band and reducibility of nitro-*H* Tyr 67 ferricytochrome *c* decreased simultaneously with the deprotonation of a group having a pK of approximately 6, in contrast to a pK of approximately 9

[3] G. M. Smith, personal communication.

for the corresponding transition of the native molecule (74, 88). This behavior is consistent with the expected drop in the pK of the *H* Tyr 67 hydroxyl group upon nitration. Presumably ionization of *H* Tyr 67, with subsequent disruption of the *H* Tyr 67-*H* Thr 78 H-bond, leads to a more open conformation of the ferricytochrome *c* molecule, which has an altered sixth ligand rendering it no longer easily reducible. In this context it is interesting to note that ferricytochrome c_2 shows essentially similar behavior with respect to both the intensity of the 695-nm band[4] and the intensity of the contact shifted *R* Met 91 methyl resonance *versus* pH to that observed with cytochrome *c*. However, the ferricytochrome c_2 NMR spectra shows an additional contact shifted resonance which is titrated at somewhat lower pH than that ascribed to the methionine methyl resonance in both cytochromes *c* and c_2, which may be indicative of the sequential ionization of tyrosines 52 and 70 in the c_2 molecule.

From the above discussion it is apparent that cytochrome *c*, in contrast with c_2, does not show protonation coupled to oxidoreduction in the physiological range, and that its alkaline pH *versus* E_m behavior is most probably due to the conversion of the native form to an unreducible conformer resulting from disruption of the *H* Met 80 heme ligand due to the ionization of *H* Tyr 67. Although *c* and c_2 show analogous pH *versus* 695-nm band intensity behavior, it might be expected that c_2 would perhaps not undergo the transition to an unreducible conformer at alkaline pH because it is structurally constrained in a closed crevice configuration in both of its oxidation states. These observations would, in any case, call into question the proposal that cytochrome *c* reduction is facilitated by perturbation of the *H* Thr 78-*H* Tyr 67 hydrogen bond system postulated to stabilize the cytochrome *c* transition state. From Figs. 5 and 6, however, it can be seen that in our proposed mechanism protonation would only be coupled to oxidoreduction after binding to the reductase and attainment of the transition state when the internal stabilizing system is brought into play. Thus, the lack of proton coupling to cytochrome *c* oxidoreduction by small ion oxidoreductants in the physiological pH range (81), the constancy of its potential in this pH range (78), and the lack of any apparent deuterium isotope effect upon the autoexchange rate (84) would appear to indicate that the reactions of cytochrome *c* with these small ion oxidoreductants do not provide valid models for the physiological mechanism of oxidoreduction, because the physiological mechanism requires prior inward rotation of the ring of *H* Phe 82 in order to attain the physiological transition state. Cytochrome c_2, by contrast, shows protonation coupled to oxidoreduction because it is statically constrained in a configuration analogous to the proposed transition state of cytochrome *c*. Indeed it has been recently observed that deuterium oxide substitution for H_2O in photosynthetic bacterial whole cells and mitochondrial membrane fragments results in an isotope effect of $\sqrt{2}$ for the ratio of cytochrome oxidation-reduction half times (89), consistent with the existence of oxidation-reduction-coupled protonation proposed to be important in the physiological oxidoreduction of cytochromes *c* and c_2.

The suggestions that oxidized cytochrome *c* may exist in a conformer in which *H* Phe 82 is pushed into the heme crevice prior to heme reduction and that it is the great increase in the *H* Met 80 sulfur-heme iron bond strength which supplies the mechanical force required for the attainment and maintenance of the over-all reduced conformation are supported by observations on the oxidoreduction of cytochrome *c* in the crystalline state. Thus, although it is possible to oxidize crystals of reduced cytochrome *c* (90, 91), attempts to reduce crystals of oxidized cytochrome *c* result in destruction of the crystals (3, 63). This implies that the crystal lattice packing forces are sufficient to maintain the molecules in their reduced conformation (with the ring of *H* Phe 82 buried), even though the heme iron is oxidized with the concomitant weakening of the *H* Met 80 sulfur-heme iron bond. However, in the reverse case, the lattice packing forces are not sufficiently strong to constrain the molecules in their oxidized configuration when the heme iron is reduced (Fig. 8). The suggestion that the increase in *H* Met 80-heme iron bond strength serves as the prime mover for the conformational transition to the reduced configuration is further implied by the hydrated electron reduction experiments with cytochrome *c*, in which it is found spectroscopically that the conformational transition to the reduced species follows an initial fast reduction of the heme iron (71, 73, see also 92).

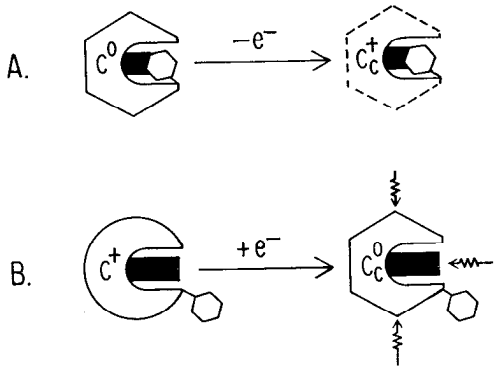


FIG. 8. Schematic of the crystalline oxidoreduction of eucaryotic cytochrome *c*. *A*, reduced ferrocytochrome *c* (C^0) may be oxidized in the crystalline state, yielding an oxidized form (C_c^+) which is constrained in the reduced molecular configuration by crystal packing forces. This implies that the closing of the heme crevice may take place prior to heme reduction, consistent with the schemes shown in Figs. 5 and 6. *B*, attempts to reduce crystals of ferricytochrome *c* result in destruction of the crystals. This implies that the strengthening of the sixth ligand-heme iron bond upon ferriheme reduction supplies the mechanical force causing the molecule to assume its reduced conformation.

Reoxidation of the reduced cytochrome *c* molecule is proposed to take place by front side approach to the oxidase which may facilitate the approach to the transition state by deprotonation of *H* Thr 78 or the abstraction of Phe 82 from the heme crevice or both. Although the deuterium isotope experiments mentioned above suggest that perturbation of the *H* Thr 78-H Tyr 67 H-bond system might play a role in the oxidation of cytochrome *c*, the poor reactivity of cytochrome c_2 (where the ring of *R* Phe 93 is constrained adjacent to the heme in both oxidation states) with the mitochondrial oxidase (43), suggests that for the case of mitochondrial cytochrome *c*, abstraction of the ring of *H* Phe 82 from the heme crevice is of primary importance in the mechanism of oxidation. In contrast, the efficiency with which cytochrome c_2 is reduced by the mitochondrial complex may be ascribed to the conformational and functional similarity between the ferricytochrome c_2 and the proposed reductase-bound form of eucaryotic cytochrome *c* (Fig. 5).

It may be argued that since cytochrome *c* which has been oxidized or reduced by physiological oxidoreductants is conformationally indistinguishable from that which is oxidized or reduced by other means (*i.e.* small ions or hydrated electrons), that conformational changes induced in cytochrome *c* upon bind-

[4] F. R. Salemme, unpublished results.

ing to the physiological oxidoreductants play a negligible role in the facilitation of the oxidoreduction mechanism. The present authors feel, however, that the high efficiency and specificity manifest by cytochrome *c* in electron transport to and from its nearly isopotential oxidase and reductase strongly support a facilitated mechanism of the type proposed above. Indeed, it may be seen there is nothing in the proposed mechanism which would suggest any difference in the final conformational states of the enzymatically oxidized or reduced molecule as compared with cytochrome *c* oxidized or reduced by other means, since it is the relative strength of the *H* Met 80 sulfur-heme iron bond in the oxidized and reduced molecule which is principally responsible for determining their conformations.

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