Review Letter

STRUCTURAL AND FUNCTIONAL FEATURES OF THE INTERACTION OF CYTOCHROME *c* WITH COMPLEX III AND CYTOCHROME *c* OXIDASE

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1. Introduction

Cytochrome *c* was the first respiratory protein of mitochondria to be isolated in pure form. The water solubility and stability of this hemoprotein has made for ease of study and cytochrome *c* is one of the best characterized proteins in the cell. The primary structure of cytochrome *c* from a multitude of different sources has been determined. The protein has been crystallized and three-dimensional structural information obtained by X-ray studies [1–3]. For tuna cytochrome *c*, the structure of the oxidized and reduced states of the protein have been determined and refined to 1.5–1.8 Å; a resolution at which bond angles and bond lengths between individual atoms can be measured [4]. In addition to these structural studies, there has been considerable work on the kinetics of electron transfer between cytochrome *c* and artificial electron donors and acceptors such as ferrocyanide [5–10]. Based on these experiments, precise mechanisms of electron transfer between small molecule electron donors and cytochrome *c* have been proposed [9–11].

The physiological electron donor and acceptor for cytochrome *c* in mitochondria are ubiquinone cytochrome *c* reductase (complex III) and cytochrome *c* oxidase (complex IV), respectively. These intrinsic membrane complexes have proved much more difficult to isolate and characterize than cytochrome *c* and only recently has it been possible to study the interaction of cytochrome *c* with either of these proteins. This review collects some of the recent observations on the binding of cytochrome *c* to complex III and cytochrome *c* oxidase and considers the implications of these results for our understanding of the terminal steps in mitochondrial electron transport.

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2. Structure of oxidised and reduced cytochrome *c*

Oxidised cytochrome *c* is a roughly spherical molecule with a diameter between 25 Å and 30 Å [4]. The heme is located in a hydrophobic pocket or cleft, extending to the surface on what Dickerson and colleagues call the front face of the protein [1]. Surrounding the heme cleft are a number of lysine residues. This ring of positive charges is conserved in all of the cytochromes *c* for which sequence data is available [12].

The structure of reduced cytochrome *c* is remarkably similar to the oxidised form [4]. Significant differences occur only near a buried water molecule which is hydrogen-bonded to Asn 52, Tyr 67 and Thr 78. This water molecule is 1.0 Å further from the heme in the reduced protein and the heme is 0.15 Å more buried in the heme crevice [4]. Thus the heme is in a less polar environment in reduced cytochrome *c*. The similarity in the structures of the oxidised and reduced forms of cytochrome *c* is consistent with the nearly identical binding constants of the two redox forms with cytochrome *c* oxidase [13,14].

3. Binding sites for complex III and cytochrome *c* oxidase on cytochrome *c*

Several approaches have been used to identify the binding sites for reductases and oxidases on cytochrome *c*. Individual lysines on cytochrome *c* have

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been modified to replace the positively charged amino group with a neutral trifluoroacetyl [15] or (trifluoromethyl) phenylcarbamoyl group [16] or with a negatively charged carboxydinitrophenyl group [17,18]. The effect of these modifications on electron transfer with reductases and oxidases has then been compared [15–18]. Modification of any of lysines 8,13,25,27,72,79,86 or 87 affected activity with either complex III or cytochrome *c* oxidase. Alteration of other lysines had a minimal effect on activity. The above-listed positively charged residues are the conserved amino acids around the heme cleft [4].

The finding that cytochrome *c* uses the same or a very similar binding site for interaction with complex III and cytochrome *c* oxidase has been confirmed by a different approach. Rieder and Bosshard have shown that the same lysine residues (8,13,72,73,86 and 87) are shielded from acetylation by complexing cytochrome *c* with either the reductase or oxidase [19,20]. Fig.1 shows the binding site for complex III and cytochrome *c* oxidase as determined by the above studies.

The same site is involved in interaction with other oxidases and reductases, for example, cytochrome *c* peroxidase [21,22], cytochrome b_5 [23] and sulfite oxidase [24,25]. The three-dimensional structure of both cytochrome b_5 [26] and cytochrome *c* peroxidase [27] is known from X-ray crystallography. It has been possible, therefore, to computer-fit the cytochrome *c*–cytochrome b_5 and cytochrome *c*–cytochrome *c* peroxidase pairs to look for sites on cytochrome b_5 and cytochrome *c* peroxidase that are complementary to the highly positively charged region on cytochrome *c* [28,29]. In both cases there is a negatively charged region complementary to the positively charged residues near the heme cleft of cytochrome *c*. The interaction between cytochrome *c* and cytochrome b_5 through these complementary binding sites would bring the two hemes within 8.5 Å of each other and in parallel orientation [28]. In the hypothetical cytochrome *c*–cytochrome *c* peroxidase complex, the hemes are 16.5 Å apart (edge to edge) and again are parallel [29].

Presumably, cytochrome c_1 of complex III and subunit II of cytochrome *c* oxidase (see later) will contain a ring of negatively charged residues which act as the binding site for cytochrome *c*.

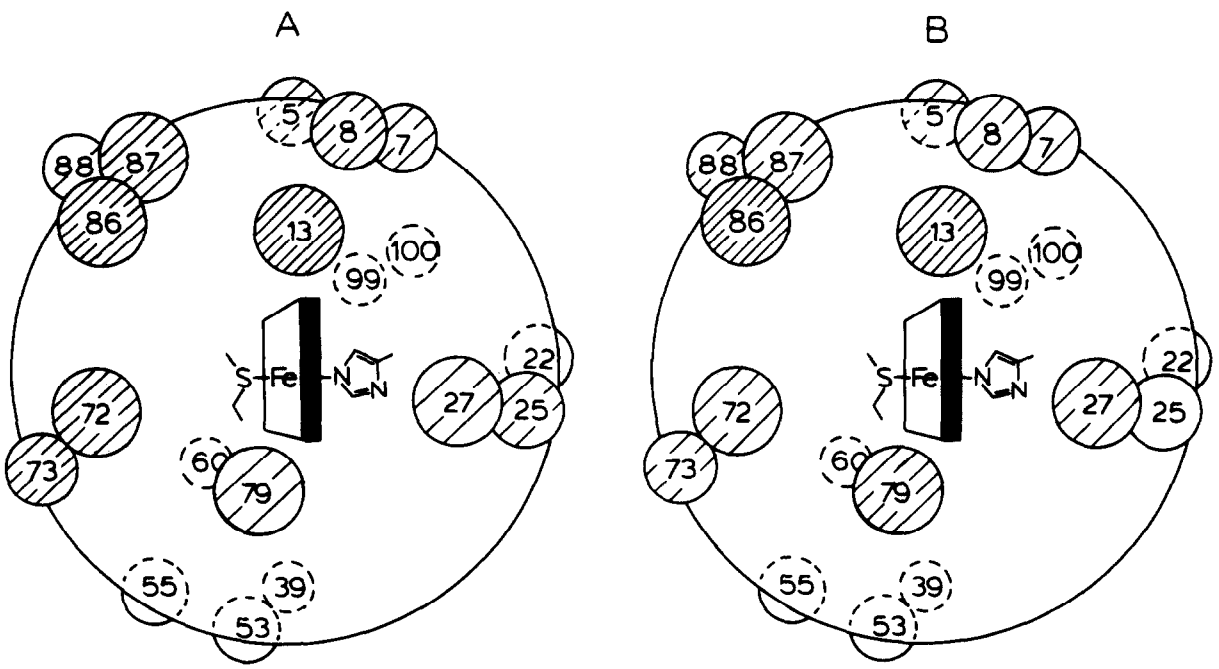

Fig.1. Schematic of the binding domain on cytochrome *c* for interaction with cytochrome *c* oxidase (A) and complex III (B). The number of diagonal hatchmarks in each circle is proportional to the contribution of that lysine to the electrostatic interaction (reproduced from [16] with permission).

4. Movement of cytochrome *c* between complex III and cytochrome *c* oxidase

The fact that the site of entry and exit of electrons to and from cytochrome *c* involves the same site argues for dissociation of cytochrome *c* from the high affinity site on the reductase before binding to the oxidase. Cytochrome *c* could remain attached to the reductase, the oxidase, or to a complex between oxidase and reductase and still transfer electrons, provided that rotational movement within a limited domain is allowed. Alternatively, cytochrome *c* could dissociate from the reductase and move to the oxidase through lateral diffusion across the surface of the membrane.

The possibility that complex III, cytochrome *c* and cytochrome *c* oxidase form a supramolecular aggregate has been examined in re-constitution experiments. Kawato et al. [30] have shown that the rate of rotational diffusion of cytochrome *c* oxidase in reconstituted vesicles is the same whether or not complex III and cytochrome *c* are present. If these components formed a stable aggregate, the rate would have been slower in the presence of complex III by a factor of 2 or 3. Kawato et al. [30] conclude that cytochrome *c* shuttles between complex III and cytochrome *c* oxidase by lateral diffusion along the membrane [30].

5. Number of binding sites and affinities for cytochrome *c* in mitochondria

Complex III has been found to contain a single binding site for cytochrome *c* per monomer with high affinity ($K_d \simeq 10^{-8}$ M) [17]. This site has been localized by chemical crosslinking to cytochrome c_1, a hemoprotein of $M_r \sim 30\,000$ [31].

The interaction of cytochrome *c* with cytochrome *c* oxidase is more complicated than with complex III. There are 2 binding sites for this substrate per cytochrome *c* oxidase monomer under conditions of low ionic strength, one with a high affinity ($K_d \simeq 10^{-8}$ M), the other with 50–100-fold lower affinity [18,32]. Both sites are important for cytochrome *c* oxidase activity as evident from kinetic studies. Fig.2 shows an Eadie–Hofstee plot of the activity of beef-heart cytochrome *c* oxidase when assayed polarographically [with *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) and ascorbate as electron donors]. Two phases of activity are seen with app. K_m 10^{-8} M and 10^{-6} M, respectively. These have been interpreted as involving electron transfer through the high and low affinity sites, respectively [18,32] (but see section 8 for alternative interpretations of the kinetic data). The lipid bilayer also provides negatively charged sites for interaction with cytochrome *c* [33]. These are of lower affinity (10^{-5} M) than sites on proteins but are in much greater numbers. Thus cytochrome *c* released from complex III and cytochrome *c* oxidase should be retained on the membrane surface rather than being free in the intracristal space.

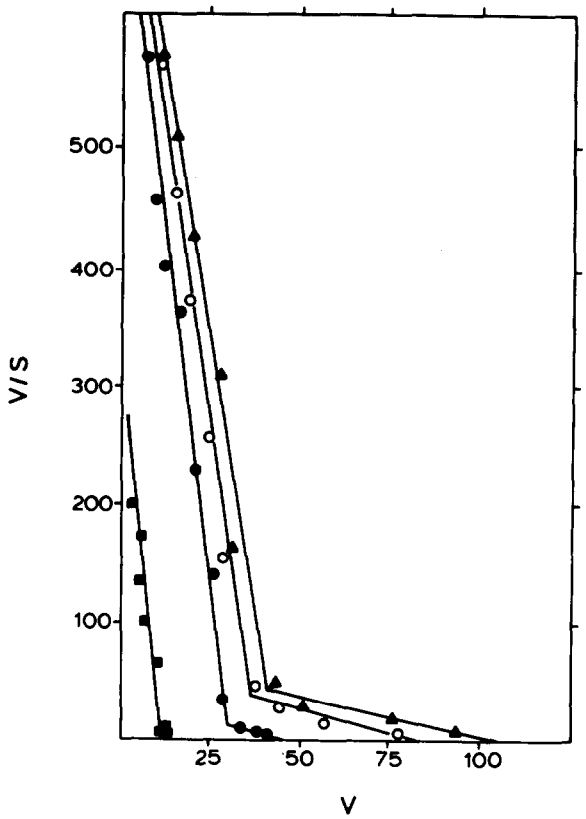

Fig.2. Eadie–Hofstee plots of cytochrome *c* oxidase activity measured polarographically with TMPD and ascorbate as electron donors. The different plots are for preparations of enzyme containing different amounts of tightly bound phospholipid as follows: (▲——▲) 30 μg/mg; (○——○) 21 μg/mg; (●——●) 10 μg/mg; (■——■) 2 μg/mg (reproduced from [45] with permission).

6. Structure of cytochrome *c* binding sites on cytochrome *c* oxidase

The locus of the high affinity site for cytochrome

c on cytochrome *c* oxidase has been characterized by crosslinking experiments [34–39]. Two cytochrome *c* derivatives have been particularly useful in this regard. One involves the modification of lysine 13 of horse heart cytochrome *c* with a photoactive aryl-azido-group, which upon illumination with UV light, inserts into a nearest nucleophilic group [36]. The arylazidocytochrome *c* derivative has been shown to bind to beef heart cytochrome *c* oxidase in the high affinity site and insert exclusively into subunit II [36,37]. Sequencing studies have further localized the site of binding of this derivative to a segment of subunit II around His 161 (R. Bisson, G. C. M. Steffens, G. Buse, R. A. C., unpublished).

The second derivative used was TNB–cytochrome *c*, a derivative of yeast cytochrome *c* in which Cys 107 has been modified by a thionitrobenzoate group [38,39]. TNB is a good leaving group and promotes disulfide bond formation between Cys 107 and any near neighbor cysteine. The TNB–cytochrome *c* derivative has been shown to bind to beef-heart cytochrome *c* oxidase in the high affinity site and link covalently in subunit III rather than subunit II [38]. TNB–Cytochrome *c* has also been covalently cross-linked to yeast cytochrome *c* oxidase through subunit III [39]. This subunit from both yeast and beef-heart cytochrome *c* oxidase contains 2 cysteines (S. Anderson, personal communication; [40]). However, only one of these is conserved and this is Cys 115 (numbering in beef heart subunit III), which is, therefore, almost certainly the site of interaction of the TNB–cytochrome *c*.

Cytochrome *c* derivatives modified at the front (Lys 13) and back (Cys 107) can only bind into the (same) high affinity site of cytochrome *c* oxidase if the site is a large cleft almost the diameter of the cytochrome *c* molecule. Subunit II must form one face of this cleft and subunit III another. A cleft of the right size is evident on the C (cytoplasmic) domain of cytochrome *c* oxidase as visualized by electron microscopy and image reconstruction studies (fig.3). This cleft is on the 2-fold axis between monomers of the cytochrome *c* oxidase dimer and each high affinity site includes subunit II of one monomer and subunit III of an opposing monomer. The interaction between the front face of cytochrome *c* and subunit II must provide the majority of the binding energy because, as discussed already, chemical modification of lysines on the back side of cytochrome *c* do not greatly affect the activity. Only when the modification of the side or back is large enough to sterically hinder the approach of cytochrome *c* to the cleft, is there a major effect, as seen for example in antibody binding experiments [41].

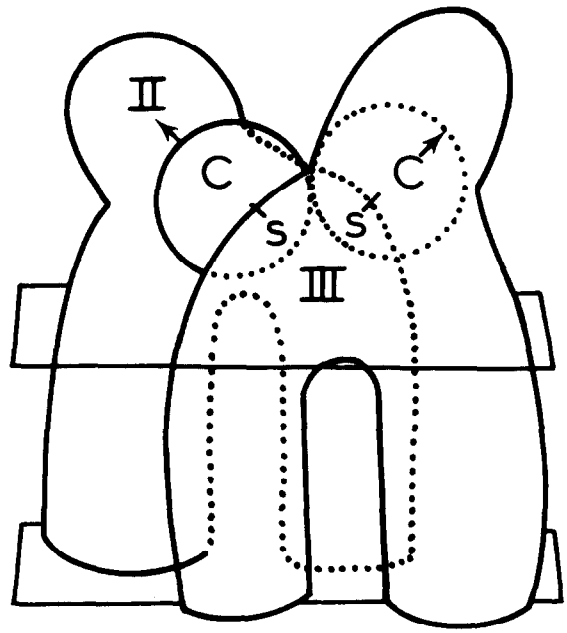

Fig.3. Schematic of the high affinity binding site(s) for cytochrome *c* in the cytochrome *c* oxidase dimer. The shape of the cytochrome *c* oxidase monomer and relationship of monomers in the dimer is taken from [43,48]. The position of 2 cytochrome *c* molecules in the 2 high affinity sites per dimer are shown. The linkage of arylazido-cytochrome *c* to subunit II is represented (→) arrow: the linkage of the TNB–cytochrome *c* to subunit III by –S.

The possibility that the high affinity site is in the interface between monomers allows an explanation of the observed 'half-of-site' effect of the arylazido-cytochrome *c* derivative on cytochrome *c* oxidase activity [37]. Similar behavior has been found for other dimeric or oligomeric proteins on binding of substrate analogues at the monomer–monomer interface and this phenomenon does not necessarily have any physiological significance [42]. Experiments with the covalently-linked yeast cytochrome *c* have shown that the monomers of the dimeric beef enzyme function independently in electron transfer [39].

Experiments are in progress to determine the locus of the high affinity binding site within the cytochrome *c* oxidase dimer more directly. Two-dimensional crystals of cytochrome *c* oxidase have been obtained using deoxycholate as a detergent in which the oxi-

dase molecules are present as monomers [43]. The arylazido-cytochrome *c* derivative has been covalently bound to these preparations without perturbing their crystalline state. Fab fragments specific for cytochrome *c* have then been bound to the lattice in order to locate the site of binding of cytochrome *c* with respect to the cytochrome *c* oxidase molecule. Preliminary results indicate that cytochrome *c* is indeed bound to that surface of the cytochrome *c* oxidase molecule which is involved in dimer formation [44]. Interestingly, the TNB–cytochrome *c* derivative does not bind to the monomeric cytochrome *c* oxidase in these crystalline preparations (S. D. Fuller, unpublished). The most likely explanation is that the specific interaction between the front face of cytochrome *c* and the oxidase molecule (on one monomer) is necessary to bring the Cys 107 in position for disulfide bond formation with subunit III (in the second monomer). (If subunits II and III of the same monomer were involved, crosslinking of the TNB–cytochrome *c* should not be affected by the aggregation state of cytochrome *c* oxidase.)

7. Identification of the low affinity site for cytochrome *c* in cytochrome *c* oxidase

Cytochrome *c* binding to beef heart cytochrome *c* oxidase has been found to depend on the lipid composition of the enzyme preparation [45]. Enzyme containing high concentrations of phospholipid ($\geqslant$20 mol/mol) binds 3 or more cytochrome *c* molecules in low ionic strength buffer and at high concentrations of cytochrome *c*. As lipid is progressively removed, the amount of cytochrome *c* that can be bound becomes lower. Cytochrome *c* oxidase containing 4 or 5 molecules of tightly bound cardiolipin binds only 2 cytochrome *c* molecules, one in the high affinity site, the second in the low affinity site ($K_d \simeq 10^{-6}$ M) [45]. As these cardiolipin molecules are progressively removed, the number of cytochrome *c* molecules bound is reduced to one and the low affinity phase of electron transfer also disappears (fig.2). These results indicate that negatively charged phospholipids contribute an important part of the low affinity binding site (see also [46]).

8. Function of the high and low affinity sites for cytochrome *c* or cytochrome *c* oxidase

The various chemical crosslinking studies have allowed the analysis of the roles of the high and low affinity sites for cytochrome *c* in cytochrome *c* oxidase activity. The covalent complexes between either arylazido-cytochrome *c* or the TNB yeast cytochrome *c* derivatives and cytochrome *c* oxidase were inactive in the spectrophotometric assay when reduced cytochrome *c* was used as a substrate [37,39]. If blocking the high affinity sites inhibits activity completely, there can be no independent electron transfer through the low affinity sites, as proposed [18,32].

Electron transfer can occur through the high affinity binding site without cytochrome *c* in the low affinity site. Lipid free cytochrome *c* oxidase retains activity although the low affinity site has been lost [43]. Also, a complex with TNB–cytochrome *c* covalently bound to cytochrome *c* oxidase in the high affinity site is active in electron transfer with artificial electron donors as substrate in the absence of cytochrome *c* in the low affinity site [39]. However, electron transfer without the low affinity site is much less than maximal, being 10–20% for lipid free enzyme and a maximum of 15% of optimal for the TNB–cytochrome *c*–cytochrome *c* oxidase complex.

We have described 3 possible mechanisms by which cytochrome *c* in the low affinity site can increase electron transfer [39]:

(1) Binding of cytochrome *c* molecules in the low affinity site decreases the affinity of cytochrome *c* in the high affinity site, so increasing the rate of electron transfer.

(2) Electron transfer proceeds through both the high and low affinity sites but with transfer through the low affinity site occurring only after an electron has entered the oxidase through the high affinity site.

(3) Binding of cytochrome *c* to the low affinity site increases the intramolecular rate of electron transfer, e.g., by increasing the rate of electron transfer from the a,Cu_a couple to the a_3,Cu_{a_3} couple.

Any of the above mechanisms would explain the 2 phases of activity seen in the Eadie–Hofstee plots shown in fig.2. Wilms et al. [47] have measured the off-rate of cytochrome *c* from the high affinity site to be 2 s^{-1} in the absence of this substrate in the low affinity site. They have shown that binding a positively charged protein in the low affinity site increases this off-rate at least 50-fold, in support of mechanism (1).

9. Summary and description of the steps in electron transfer from complex III to cytochrome *c* oxidase

The studies reviewed above suggest a model for electron transfer from complex III to cytochrome *c* oxidase with the following steps:

(i) Reduced cytochrome *c* dissociates from complex III before it can bind to cytochrome *c* oxidase. This follows from the fact that cytochrome *c* has the same binding domain for reductases and oxidases.

(ii) Electron transfer from complex III to cytochrome *c* oxidase occurs by lateral diffusion of cytochrome *c* across the surface of the membrane.

(iii) Reduced cytochrome *c* transfers into the low affinity site of cytochrome *c* oxidase which involves tightly bound cardiolipin.

(iv) Binding of cytochrome *c* in the low affinity site then effects the release of cytochrome *c* from the high affinity site either by charge repulsion or by conformational rearrangements in the cytochrome *c* oxidase molecule (mechanism (1) from section 8).

(v) Reduced cytochrome *c* in the low affinity site transfers into the high affinity site and gives up its electron to cytochrome *c* oxidase. Oxidized cytochrome *c* would be replaced by another reduced cytochrome *c* and the process occurs 4 times for each turnover of cytochrome *c* oxidase in the overall reaction:

$$4\,H^+ + 4\,\text{cytochrome } c^{2+} + O_2 \rightleftarrows 2\,H_2O + 4\,\text{cytochrome } c^{3+}.$$

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References

[1] Dickerson, R. E. and Timkovich, R. (1975) in: The Enzymes (Boyer, P. D. ed) 3rd edn, vol. 11, pp. 397–547, Academic Press, New York.
[2] Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O. B., Samson, L., Cooper, A. and Margoliash, E. (1971) J. Biol. Chem. 246, 1511–1535.
[3] Tanaka, N., Yamane, T., Tsukihara, T., Ashida, T. and Kakudo, M. (1975) J. Biochem. 77, 147–162.
[4] Takano, T. and Dickerson, R. E. (1980) Proc. Natl. Acad. Sci. USA 77, 6371–6375.
[5] Ahmed, A. J. and Millett, F. (1981) J. Biol. Chem. 256, 1611–1615.
[6] Brandt, K. G., Parks, P. C., Czerlinski, G. H. and Hess, G. P. (1965) J. Biol. Chem. 241, 4180–4185.
[7] Cassatt, J. C. and Marinii, C. P. (1974) Biochemistry 13, 5323–5328.
[8] Ilan, Y. and Schaffermann, A. (1979) Biochim. Biophys. Acta 548, 161–165.
[9] Marcus, R. A. and Sutin, A. (1975) Inorg. Chem. 14, 213.
[10] Stellwagen, E. and Shulman, R. G. (1973) J. Mol. Biol. 80, 559–573.
[11] Wherland, S. and Gray, H. B. (1977) in: Biological Aspects of Inorganic Chemistry (Addison, A. W. ed) pp. 289–368, Wiley, New York.
[12] Dickerson, R. E., Timkovich, R. and Almassy, J. (1976) J. Mol. Biol. 100, 473–491.
[13] Hill, B. C. and Nicholls, P. (1980) Biochem. J. 187, 809–818.
[14] Peterson, L. C. (1978) FEBS Lett. 94, 105–108.
[15] Staudenmayer, N., Ng, S., Smith, M. B. and Millett, F. (1977) Biochemistry 16, 600–604.
[16] Smith, H. T., Ahmed, A. J. and Millett, F. (1981) J. Biol. Chem. 256, 4984–4990.
[17] Speck, S. H., Ferguson-Miller, S., Osheroff, N. and Margoliash, E. (1979) Proc. Natl. Acad. Sci. USA 76, 155–159.
[18] Ferguson-Miller, S., Brautigan, D. L. and Margoliash, E. J. (1978) J. Biol. Chem. 253, 149–159.
[19] Reider, R. and Bosshard, H. R. (1980) J. Biol. Chem. 255, 4732–4739.
[20] Reider, R. and Bosshard, H. R. (1978) J. Biol. Chem. 253, 6045–6051.
[21] Kang, C. H., Brautigan, D. L., Osheroff, N. and Margoliash, E. (1978) J. Biol. Chem. 253, 6502–6510.
[22] Smith, M. B. and Millett, F. S. (1980) Biochim. Biophys. Acta 626, 64–72.
[23] Stonehuerner, J., Williams, S. B. and Millett, F. S. (1979) Biochemistry 18, 5422–5427.
[24] Webb, M., Stonehuerner, I. and Millett, F. S. (1980) Biochim. Biophys. Acta 543, 290–298.
[25] Speck, S. H., Koppenol, W. H., Dethmers, I. K., Osheroff, N., Margoliash, E. and Rajagopalan, K. V. (1981) J. Biol. Chem. 256, 7394–7402.
[26] Mathews, F. S., Argos, P. and Levine, M. (1971) Cold Spring Harbor Symp. Quant. Biol. 36, 387–395.
[27] Poulos, T. L., Freer, S. T., Alden, R. A., Edwards, S L., Skogland, V., Takio, K., Eriksson, B., Xuong, N., Yonetani, T. and Kraut, J. (1980) J. Biol. Chem. 255, 575–580.
[28] Salemme, F. R. (1976) J. Mol. Biol. 102, 563–568.
[29] Poulos, T. L. and Kraut, J. (1980) J. Biol. Chem. 255, 10,322–10,330.
[30] Kawato, S., Sigel, E., Carafoli, E. and Cherry, R. J. (1981) J. Biol. Chem. 256, 7518–7527.

[31] Bruger, C., Nalecz, M. J. and Azzi, A. (1980) Biochim. Biophys. Acta 592, 510–527.
[32] Ferguson-Miller, S., Brautigan, D. L. and Margoliash, E. (1976) J. Biol. Chem. 251, 1104–1115.
[33] Birrell, B. and Griffith, O. H. (1976) Biochemistry 15, 2925–2929.
[34] Briggs, M. and Capaldi, R. A. (1978) Biochem. Biophys. Res. Commun. 80, 553–559.
[35] Seiter, C. H. A., Margalit, R. and Perrault, R. (1979) Biochem. Biophys. Res. Commun. 86, 473–477.
[36] Bisson, R., Azzi, A., Gutweniger, H., Colonna, R., Montecucco, C. and Zanotti, A. (1978) J. Biol. Chem. 253, 1874–1880.
[37] Bisson, R., Jacobs, B. and Capaldi, R. A. (1980) Biochemistry 19, 4173–4178.
[38] Birchmeir, W., Kohler, C. E. and Schatz, G. (1976) Proc. Natl. Acad. Sci. USA 73, 4334–4338.
[39] Fuller, S. D., Darley-Usmar, V. M. and Capaldi, R. A. (1982) Biochemistry in press.
[40] Thalenfield, B. E. and Tzagoloff, A. (1980) J. Biol. Chem. 255, 6173–6180.
[41] Osheroff, N., Jemmerson, R., Speck, S. H., Ferguson-Miller, S. and Margoliash, E. (1979) J. Biol. Chem. 254, 12717–12724.
[42] Levitzki, A. and Koshland, D. E. (1976) Curr. Top Cell. Reg. 10, 1–38.
[43] Fuller, S. D., Capaldi, R. A. and Henderson, R. (1979) J. Mol. Biol. 134, 305–327.
[44] Fuller, S. D. (1980) PhD Thesis, University of Oregon.
[45] Vik, S. B., Georgevich, G. and Capaldi, R. A. (1981) Proc. Natl. Acad. Sci. USA 78, 1456–1460.
[46] Robinson, N. C., Strey, F. and Talbert, L. (1980) Biochemistry 19, 3656–3660.
[47] Wilms, J., Veerman, E. C. I., Konig, B. W., Dekker, H. L. and Van Gelder, B. F. (1981) Biochim. Biophys. Acta 635, 13–24.
[48] Deatherage, J., Capaldi, R. A. and Henderson, R. (1982) J. Mol. Biol. in press.