Electron Transfer Reactions in Biological Systems: The Reduction of Ferricytochrome *c* by Chromous Ions

[Cr(III)-ferrocytochrome *c* complex/crosslinkage]

CAROL J. GRIMES, DENNIS PISZKIEWICZ, AND EVERLY B. FLEISCHER

Departments of Chemistry and Biological Chemistry, University of California at Irvine, Irvine, Calif. 92664

Communicated by Harry B. Gray, January 10, 1974

ABSTRACT Chromous ion reacts with ferricytochrome *c* to yield a one-to-one Cr(III)-ferrocytochrome *c* complex. This material, when hydrolyzed by trypsin and subjected to chromatographic procedures, yielded two fragments containing chromium. The amino-acid compositions and chemical characteristics of each of these fragments indicated that the chromium had crosslinked two segments of polypeptide chain; these were residues 40-53-Cr(III)-residues 61-72 and residues 40-53-Cr(III)-residues 61-73. Examination of a model of the ferricytochrome *c* molecule indicated that only two residues of the crosslinked peptides were sufficiently close to allow crosslinking to take place. These residues were tyrosine 67 and asparagine 52. Enzymatic hydrolysis of one of those fragments by aminopeptidase M supported this identification. The position of the chromic ion implies what is the path of electron transfer from the chromous ion to the ferric ion in this chemical reduction of cytochrome *c*, and suggests a possible path of electron transfer in biological oxidation-reduction reactions.

The various components of the mitochondrial electron transport system have been characterized, their order of reactivity has been ascertained, and some of their molecular properties have been defined (1-3). The general outlines of the electron transport chain are understood, but the detailed mechanism by which electron transfer (or atom transfer) takes place between the various components of the respiratory chain is not well understood. We report the results of studies of systems that will lead to some insight into the details of electron transfer in cytochrome *c*.

Cytochrome *c* is a well-understood component of the electron transport chain (4). The structures of both the oxidized and reduced cytochrome *c* have been determined (5, 6). Kowalsky studied the reduction of ferricytochrome *c* with chromous ions and demonstrated that the inert Cr(III) product was tightly bound to the cytochrome *c* moiety (7); several groups have studied the kinetics of this reaction (8, 9). The chemistry of the chromic ion is characterized by its substitutional inertness (10); thus, the product of this reaction which has Cr(III) bound to cytochrome *c* should be relatively stable and amenable to chemical studies of its structure. This paper reports the location of the chromic ion in the chromic-cytochrome *c* complex, and considers the implications regarding the mechanism of electron transfer in cytochrome *c*.

MATERIALS AND METHODS

Materials. Horse-heart cytochrome *c* (type III) was obtained from Sigma Chemical and was used without further purification. Trypsin treated with L-(tosylamido-2-phenyl)

ethyl chloromethyl ketone was obtained from Worthington Biochemical Co. Aminopeptidase M was from Henley and Co. of New York. Sephadex G-50 superfine and G-25 superfine were from Pharmacia. Dowex 50-X2 resin (AG50W-X2) and Dowex-1-chloride (AG1-X8) were supplied by Bio-Rad, Richmond, Calif. ICN, Irvine, Calif., was the source of the $^{51}\text{Cr}(\text{III})$ used to generate $^{51}\text{Cr}(\text{II})$. The $^{51}\text{Cr}(\text{III})$ was obtained in a solution of 0.5 N HCl, and was diluted with H_2O 1000-fold before use. All other chemicals were of the highest quality available. Nitrogen gas from Matheson was passed through scrubbing towers of chromous solution before use in reaction vessels.

Preparation of Cr(III)-Cytochrome *c*. Chromous solutions for reduction of the protein were prepared by reducing a deoxygenated solution of about 10 mM $\text{Cr}(\text{ClO}_4)_3$ in 0.1 mM HClO_4 with Zn(Hg), under an atmosphere of nitrogen. Whenever radioactive labeling was desired, up to 250 μCi of $^{51}\text{Cr}(\text{III})$ in solution was added before generation of Cr(II).

The procedure for labeling the cytochrome *c* with chromic ion follows that of Kowalsky (7) in essence, but differs in details. To a solution of cytochrome *c* (20-40 mg/ml of H_2O , 50-200 mg total) was added 20 mole % $\text{K}_3\text{Fe}(\text{CN})_6$ to ensure that all the protein was in the oxidized, or ferric, form. This solution was passed through a column of Dowex-1-chloride (1×10 cm) (7), and the visible spectrum checked for the presence of only the ferric form. The pH of the solution was adjusted to 4.5, and the volume adjusted to 25 ml. An aliquot was then analyzed for protein concentration, using the magnitude of the absorbance at 528 nm [$\epsilon = 11200$ (11)].

The concentration of the chromous ion in the reducing solution was determined by the change in absorbance of a deoxygenated permanganate solution with and without added chromous ion [$\lambda_{\text{max}} = 545$ nm, $\epsilon = 2340$ (9)]. A stoichiometric amount of chromous ion was added to the deoxygenated protein solution, which was maintained under nitrogen at 0°. The chromous solution must be added slowly, with agitation of the protein solution, in order to effect a 1:1 complex of Cr(III) with cytochrome *c*. The labeled protein was passed through a Dowex-50 column to remove any chromium not bound to the protein; excess Cr(III) can be completely separated from ferricytochrome *c* by either ion exchange or dialysis procedures. All experiments reported here were done with labeled Cr(III)-cytochrome *c* having a chromium/protein ratio of 0.8-1.0. This ratio of chromium binding is pH-dependent, with the maximum ratio of 1.0 occurring at a pH of 4.0. Thus, the binding ratio of 1.0, which was previously

noted (7) to be attainable only in the presence of phosphate, has now been demonstrated to be a pH effect. (At pH = 6.0 the binding ratio is about 0.5.)

Analyses. The protein concentration was estimated by the absorbance at 410 nm [isosbestic point, $\epsilon = 106.1 \times 10^3$ (11)]. Chromium was determined by the diphenylcarbazide method after oxidation of the chromium to chromium (VI) with ammonium persulfate and silver nitrate (12). The protein solution was decomposed by addition of two drops of concentrated HNO_3 , followed immediately by oxidation with persulfate.

Hydrolysis by Trypsin. Labeled protein was hydrolyzed by trypsin at room temperature (22°) immediately after denaturation of the cytochrome *c* with trichloroacetic acid (5%). After the protein had been suspended in water at a concentration of 2–4 mg/ml, the pH was adjusted to 8.0, and 2% by weight of trypsin was added. The pH was maintained at 8.0 by addition of 0.05 N NaOH. When the rate of consumption of base neared zero, another 2% by weight of trypsin was added, and the hydrolysis was continued until no more base was consumed (3–4 hr total). Glacial acetic acid was added to lower the pH to below 3, and the sample was evaporated to dryness.

Column Chromatography. Columns of both Sephadex G-50 superfine (2.5 × 140 cm) and G-25 superfine (2.5 × 140 cm) were used in separation of the tryptic peptides. The eluant was 0.1 M acetic acid, and ambient temperatures were either 22° or 4°. Polypeptide fractions were located by the ninhydrin method after alkaline hydrolysis (13). Other methods used in sequencing are given elsewhere (14, 14a).

Amino-Acid Analysis. Samples were hydrolyzed in evacuated glass tubes at 110° for 24 hr with 6 N HCl containing 1 drop of 1% phenol in water. Analyses were performed with a Beckman model 121 automatic amino-acid analyzer.

Physical Measurements. Visible-UV spectra were collected with a Beckman Acta III Spectrophotometer. Radioactivity of samples was measured with a Tracerlab Versamatic II scaler with a NaCl well-type gamma counter. Activation analysis was performed at the UCI TRIGA Reactor complex; the standard chromium solution was prepared from 99.99% pure chromium metal.

Sedimentation Velocity Experiments. Samples in water were dialyzed exhaustively against 1 mM Tris–0.1 M KCl. The concentrations of samples to be compared were adjusted to be equal. Sedimentation velocity measurements were made with a Beckman model E Ultracentrifuge at 56,000 rpm and 20°.

RESULTS

Cr(III)–Cytochrome *c*. Ferricytochrome *c*, when reduced by chromous ion, consistently gave products with Cr:protein ratios of 0.8–1.0. Kowalsky (7) has reported that he was unable to achieve 1:1 adducts of Cr(III) to cytochrome *c* without the introduction of phosphate ions into the solution. In our experiments the presence of additional anions was not necessary. We attribute this to our rigorous attempts to exclude oxygen from our reaction mixture which could oxidize Cr(II) before reaction with cytochrome *c* and to proper control of the pH. We have taken samples of the Cr(III)–ferricytochrome *c* and oxidized it with ferricyanide and re-reduced the complex

without loss of chromium. Thus, many cycles of oxidation–reduction can be carried out on the chromium-labeled protein without loss of the chromium.

Chromium–Protein Bond. In order to establish the strength and covalency of the Cr(III)–protein bond, an aliquot of the radioactively labeled Cr(III)–cytochrome *c* was first denatured with 6 M guanidine·HCl. The protein solution was adjusted to pH 8, and then dialyzed against 10 mM phosphate buffer (pH = 7). After dialysis the contents of the dialysis bag and the external buffer were counted. The ratio of cpm/mg of protein remained unchanged by dialysis; the radioactivity of the external buffer remained at background level. Therefore, Cr(III) must be covalently bonded to cytochrome *c*.

Integrity of the Iron–Methionine 80 Sulfur Bond. The 695-nm band in the visible spectrum of ferricytochrome *c* is generally attributed (31) to an iron–sulfur charge transfer process, the sulfur being the sixth position ligand contributed by methionine 80. To determine whether or not the Cr binds directly to the iron, displacing this sulfur, some of the Cr(III)–cytochrome *c* was reoxidized with $\text{K}_3\text{Fe}(\text{CN})_6$ and the 695-nm band was examined. The band was present and had suffered no reduction in extinction coefficient. This experiment indicates no large change in conformation of the protein has taken place during the formation of the Cr(III)–ferricytochrome *c*.

Identification of the Labeled Peptide. The Cr(III)–cytochrome *c* product was hydrolyzed with trypsin, and the resultant mixture of peptides was fractionated by the column chromatographic methods described below.

An initial attempt to separate the peptides on Dowex-50 with a pH gradient by a standard method (14) was made. The chromium came through the column at the void volume, unassociated with any polypeptides, and a normal pattern of peptides followed (15). We concluded that the Cr–protein bond is decomposed by the pyridine–acetate buffers used to establish the gradient. From this experiment a pure peptide composed of residues 61–72 (see Fig. 2) was isolated and used in a study described below: Glu, 4.4(4); Thr, 0.79(1); Leu, 2.2(2); Met, 0.94(1); Tyr, 0.96(1); Asp, 1.0(1); Pro, 0.98(1); Lys, 1.0(1).

In the second attempt at fractionation, the tryptic peptides were passed through a column of Sephadex G-50 superfine (2.5 × 140 cm) at 22°. The peptide-containing fractions eluted from the column were identified by reaction with ninhydrin after alkaline hydrolysis, and the fractions containing the radioactive ^{51}Cr coincided with peptide-containing regions (Fig. 1a). Electrophoresis of the pooled, radioactive fractions at pH 1.9 yielded four peptides, the least mobile of which contained the radioactive label. Amino-acid analysis (Table 1) showed that this material, designated Fragment I, was composed of an equimolar mixture of residues 40–53 and 61–72 (see Fig. 2).

In the final fractionation experiment the tryptic peptides were separated on a column of Sephadex G-25 superfine (2.5 × 140 cm) at 4°. The elution pattern showed multiple radioactive peaks, probably due to incomplete hydrolysis (Fig. 1b). This material was pooled into three radioactive fractions and subjected to preparative paper electrophoresis at pH 1.9. The only radioactively labeled peptide isolated was from the major radioactive fraction. This material had the amino-acid composition of residues 40–53 and 61–73 (see Fragment II, Table 1); it was identical in composition with the crosslinked

FIG. 1. Peptide maps. Elution profiles of tryptic peptides from: (a) Sephadex G-50 Superfine (2.5 × 140 cm) and (b) Sephadex G-25 Superfine. Ten ml per tube were collected. Absorbance, 570 nm (—); cpm (---); background level (---). Solid bars represent pooled fractions.

material described above (residues 40–53 and 61–72) with an additional lysyl residue. On the basis of the specificity of trypsin and the known amino-acid sequence of cytochrome *c*, this residue must be at position 73 (Fig. 2). Activation analysis for Cr in Fragment II indicated a Cr:crosslinked peptide ratio of 1.3.

Evidence for Crosslinking of these peptides rather than an equimolar mixture of them comes from several experiments.

(1) Electrophoresis of Fragment I at both pH 1.9 and 4.7 resulted in a chromatogram containing only one spot. At pH 1.9 it ran with aspartic acid, while at pH 4.7 it ran slightly farther toward the anode than glutamic acid. A pure sample of the unlabeled peptide composed of residues 61–72 ran at the same position as the labeled peptide at pH 1.9, but ran behind glutamic acid at pH 4.7. Thus, Fragment I could not be composed of equimolar quantities of residues 40–53 and 61–72.

(2) In our earlier experiments any Cr-peptide products from tryptic hydrolysis were decomposed to Cr plus peptides in a pyridine-acetate buffer. Fragment I was incubated in 2 M pyridine-acetate buffer for 18 hr at 40°. Electrophoresis of this material at pH 4.7 showed multiple components. One of these components migrated to about the same position as peptide 61–72 and had the same amino-acid composition when examined by electrophoresis at pH 1.9 after acid hydrolysis. A second component, which stayed near the origin, had the composition of residues 40–53 when examined by electrophoresis at pH 1.9.

FIG. 2. Amino-acid sequence of horse heart cytochrome *c* (see ref. 23).

TABLE 1. Amino-acid compositions of labeled polypeptides

	Fragment I	Fragment II	Residues 40-53 + 61-72(73)
Aspartic acid	3.2	3.1	3
Threonine	3.2	3.0	4
Serine	—	—	—
Glutamic acid	4.8	4.6	5
Proline	1.8	1.8	2
Glycine	2.6	2.3	2
Alanine	2.1	2.2	2
Valine	0.46	—	—
Methionine	0.82	0.73	1
Isoleucine	—	0.32	—
Leucine	1.9	1.9	2
Tyrosine	1.8	1.8	2
Phenylalanine	1.0	1.0	1
Histidine	—	—	—
Lysine	2.0	3.0	2(3)
Arginine	—	—	—
Yield = 17.3%		Yield = 4.0%	

Proof of Inter- Compared to Intramolecular Crosslinking. The question of any intermolecular bonding of cytochrome *c* molecules compared to the proposed intramolecular crosslink was settled by the following experiment. Samples of both the labeled and unlabeled protein were subjected to ultracentrifugation for sedimentation velocity studies. Qualitatively, only one peak was seen for each of the samples. Quantitatively, the sedimentation constant $s_{20, w}$ was 1.66 for cytochrome *c* and 1.55 for Cr(III)-cytochrome *c*. The value of $s_{20, w}$ is 1.83 (17) for monomeric equine cytochrome *c*, and is 2.64 for the dimer of equine cytochrome *c* (16). These results show that the labeled protein is a monomer rather than a dimer or oligomer, as would be the case for intermolecular crosslinks.

Identification of the Bonded Amino-Acid Residues. Having discovered the region of the amino-acid sequence to which the chromium atom was bound, we wished to find out exactly which amino-acid residues were involved in the crosslink. Inspection of a model of the oxidized form of cytochrome *c* [built from coordinates supplied by Dickerson (17)] shows that in the absence of any gross conformational changes in the protein there are a limited number of possibilities for the amino-acid residues involved in a crosslink. The closest distance of approach between the two chains occurs between tyrosine 67 and asparagine 52, both of which are suitable

ligands for binding chromium. The distance between these two residues is about 4.9 Å, and the path between them is unobstructed by other amino-acid residues. The dimensions of this cavity are certainly adequate for the inclusion of chromium, and the distance between the two residues is admirably suited for a ligand-Cr-ligand interaction. Only one other crosslink appears possible, i.e., tyrosine 67-Cr-tyrosine 48, although this would necessitate a certain degree of strain and rearrangement to accommodate the bond. All other amino-acid side-chain ligand combinations (Table 2) are too far from each other to act as ligands in a crosslink to chromic ion without large structural changes in the protein.

Hydrolysis by Aminopeptidase M. An aliquot of Fragment I was hydrolyzed with aminopeptidase M. Under normal circumstances this exopeptidase could be expected to digest both peptides 40-53 and 61-72 completely, except for the alanine 43-proline 44 bond, which is resistant to cleavage by this enzyme (30). The results of amino-acid analysis are: Thr + Asn, 3.4; Gly, 1.4; Glu, 1.7; Ala, 0.99; Pro, 0.19; Phe, 0.87; Tyr, 1.3; Asp, 1.1; Lys, 1.1; Leu, 0.96. These results can be interpreted as follows. Residues 61-64 are present, and residues 40-53 with two exceptions: alanine 43-proline 44 (enzymic exclusion), and one threonine or asparagine. Since the analysis was done in sodium citrate buffers at 55° (18), asparagine was not separable from threonine. There are a total of three threonines and one asparagine in residues 40-53, and one threonine in 61-64, giving a total of five residues. The amino-acid analysis shows a maximum of four residues at the threonine-asparagine position. Thus, the results of aminopeptidase M hydrolysis of the crosslinked peptide agrees with the concept of a crosslink by Cr(III) of asparagine 52 and tyrosine 67. The fact that amino-peptidase M released the Cr(III)-bound asparagine 52 but stopped at the residue preceding Cr(III)-bound tyrosine 67 is not understood.

In our initial separation experiment, the Cr(III) crosslinked amino-acid bonds were found to be unstable under conditions where peptide bonds are stable, e.g., in pyridine-acetate buffers. Furthermore, the instability of this material was suggested by the relatively low yields of the labeled fragments finally isolated (Table 1). Thus, approaches to identification of the crosslinked residues by techniques such as the Edman degradation did not seem promising, and further chemical characterization of the crosslinked material was not attempted.

DISCUSSION

Chromous ion reduces ferricytochrome *c* with the resultant formation of a one-to-one complex of chromic ion and ferro-

TABLE 2. Various distances in the Cr(III)-cytochrome *c* complex

Cr(III)-Fe(III)	6.8 Å	Tyr 67-Thr 47	13.8 Å
Cr(III)-Met 80S	6.1 Å	Tyr 67-Thr 49	7.7 Å
Cr(III)-pyrrole C	5.4 Å	Tyr 67-Asp 50	14.7 Å
Cr(III)-Tyr 67	2.4 Å	Asp 70-Asp 52	16.0 Å
Tyr 67-Asn 52	4.9 Å	Glu 66-Asp 52	15.5 Å
Tyr 67-Tyr 48	8.0 Å	Met 65-Tyr 48	19.7 Å
Tyr 67-Thr 40	10.8 Å	Thr 40-Asp 62	12.4 Å
Tyr 67-Asp 41	11.7 Å		

Distances from possible sidechain ligands (i.e., O, N, S). From ref. 17.

cytochrome *c*. A chemical analysis of the product and steric considerations dictated by the three-dimensional structure of ferricytochrome *c* (12) indicates that chromium crosslinks tyrosine 67 and asparagine 52. Although we indicate the chromium is crosslinked in the same position in the isolated peptide fragment, in the chromium-ferrocyclochrome *c* complex, and, most important to our argument concerning electron-transfer, in the chromous-ferricytochrome *c* complex, we cannot definitely prove that the chromous reduction takes place without any movement of the chromium ion after reduction. Our interpretation of the results is based on the known chemistry of the chromic ion plus our control experiments; we feel our interpretation is most reasonable.

An examination of the three-dimensional structure of ferricytochrome *c* clearly shows that the chromic ion assumes a position in a pocket in the interior of the globular protein. Fig. 3 shows a three-dimensional view of the two polypeptide chains crosslinked by chromium plus the heme moiety. It was drawn assuming that no change of the protein conformation took place upon chromic complexation. Because of the cross-linked nature of the chromium bonds there is little room for movement of the chromium without considerable distortion of the entire protein structure. Since chromic ion is usually coordinated by six ligands, the coordination sphere must be completed by four water molecules in addition to the tyrosine oxygen and asparagine nitrogen.

Both residues tyrosine 67 and asparagine 52 are invariant in the sequences of all cytochromes *c* determined (20), except that of *Euglena gracilis* (20, 21) in which tyrosine 67 is replaced by a phenylalanine. Furthermore, in equine cytochrome *c*, tyrosine 67 and tyrosine 74 are selectively iodinated by KI₃ (22), while tyrosine 67 and tyrosine 48 are selectively nitrated by tetranitromethane (23, 24). Both of these modifications result in loss of activity by the protein, and prove the

FIG. 3. Three-dimensional drawing of the chromium crosslinked peptide and the heme group to illustrate their relative positions.

accessibility of the tyrosine 67 in native cytochrome *c* to fairly large molecules.

We propose a mechanism for electron transfer in the reduction of cytochrome *c* by chromous ion which is suggested by the position of tyrosine 67 in the tertiary structure of the protein. The phenyl ring of the tyrosine is nearly parallel to the plane of the heme in the oxidized form of cytochrome *c* (Fig. 3), and about 4-Å distant. The chromous ion would first coordinate to the tyrosine oxygen, then transfer an electron to the heme via the overlapping π clouds of the tyrosine and porphyrin, and then through the conjugated system of the porphyrin to the iron.

This model study shows the possibility of certain pathways for the reduction of cytochrome *c* that have been speculative. For example, on the basis of model studies, Winfield (27, 28) proposed a mechanism that would involve the participation of a phenylalanine or tyrosine. Since these residues can be induced to form free radicals—and, consequently carry an electron—one of these residues in proximity to the heme could act as an intermediary between the oxidizing agent and the metal ion. An exact mechanism for this interaction (i.e., transfer of the electron through overlapping π clouds) was not proposed.

Dickerson and his coworkers (25, 26) have recognized the possible significance of tyrosine 67 in the electron transfer reaction on the basis of its position in the tertiary structure of the protein. They have speculated that in the *in vivo* reduction of cytochrome *c*, the electron may be transferred from other residues to tyrosine 67.

In conclusion, this study indicates the participation of tyrosine 67 in the reduction of ferricytochrome *c* by chromous ion and suggests the participation of this residue as part of the reduction mechanism *in vivo*.

This work was supported by a grant from the National Science Foundation (GP-30660).

1. *The Molecular Basis of Electron Transport* (1972) eds. Schultz, J. & Cameron, B. F. (Academic Press, New York).
2. *Electron and Coupled Energy Transfer in Biological Systems* (1971) eds. King, T. E. & Klingenberg, M. (Marcel Dekker, Inc., New York), Vol. I, Parts A and B.
3. *Probes of Structure and Function of Macromolecules and Membranes* (1971) eds. Chance, B., Yonetani, T. & Mildvan, A. (Academic Press, New York), Vol. II.
4. Margoliash, E. & Schejter, A. (1966) *Advan. Protein Chem.* **21**, 113–286.
5. Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O., Samson, L., Cooper, A. & Margoliash, E. (1971) *J. Biol. Chem.* **246**, 1511–1535.
6. Dickerson, R. E., Takano, T. & Kallai, O. B. (1971) *Cold Spring Harbor Symp. Quant. Biol.* **36**, 397–404.
7. Kowalsky, A. (1969) *J. Biol. Chem.* **244**, 6619–6625.
8. Yandell, J. K., Fay, D. P. & Sutin, N. (1973) *J. Amer. Chem. Soc.* **95**, 1131–1137.
9. Dawson, J. W., Gray, H. B., Holwerda, R. A. & Westhead, E. W. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 30–33.
10. Taube, H. (1970) *Electron Transfer Reactions of Complex Ions* (Academic Press, New York).
11. Margoliash, E. & Frohwirt, N. (1959) *Biochemistry* **71**, 570–572.
12. Sandell, E. B. (1959) in *Colorimetric Determination of Traces of Metals* (Interscience Publishers, New York), p. 392.
13. Hirs, C. H. W., Moore, S. & Stein, W. H. (1956) *J. Biol. Chem.* **219**, 623–630.
14. Landon, M., Melamed, M. D. & Smith, E. L. (1971) *J. Biol. Chem.* **246**, 2360–2373.
- 14a. Landon, M., Piszkiwicz, D. & Smith, E. L. (1971) *J. Biol. Chem.* **246**, 2374–2399.
15. McDowall, M. A. & Smith, E. L. (1965) *J. Biol. Chem.* **240**, 4635–4647.
16. Margoliash, E. & Lustgarten, J. (1962) *J. Biol. Chem.* **237**, 3397–3405.
17. Dickerson, R. E., private communication.
18. Spackman, D. H., Stein, W. H. & Moore, S. (1958), *Anal. Chem.* **30**, 1190–1198.
19. Dickerson, R. E. (1972) *Sci. Amer.* **226**, 58–72.
20. Pettigrew, G. W. (1973) *Nature* **241**, 531–533.
21. Lin, D. K., Niece, R. L. & Fitch, W. M. (1973) *Nature* **241**, 533–535.
22. McGowan, E. B. & Stellwagen, E. (1970) *Biochemistry* **9**, 3047–3052.
23. Skov, K., Hofmann, T. & Williams, G. R. (1969) *Can. J. of Biochem.* **47**, 750–752.
24. Schejter, A. & Sokolovsky, M. (1969) *FEBS Lett.* **4**, 269–272.
25. Dickerson, R. E., Takano, T., Kallai, O. B. & Samson, L. (1972) in *Structure and Function of Oxidation Reduction Enzymes*, eds. Akeson, A. & Ehrenberg, A. (Pergamon Press, New York), pp. 69–83.
26. Dickerson, R. E., Takano, T. & Kallai, O. B. (1972) "Chain Folding, Reduction Mechanism, and Redox Potential in Cytochrome *c*," in *Fifth Jerusalem Symposium*, eds. Pullman, B. & Bergmann, E. D. (Israel Academy of Sciences and Humanities, Academic Press, New York).
27. Winfield, M. E. (1964) *Oxidases and Related Redox Systems*, eds. King, T. E., Mason, H. S. & Morrison, M. (Wiley, New York).
28. Winfield, M. E. (1965) *J. Mol. Biol.* **12**, 600–604.
29. Smith, L., Davies, H. C., Reichlin, M. & Margoliash, E. (1973) *J. Biol. Chem.* **248**, 237–243.
30. DeLange, R. J. & Smith, E. L. (1971) in *The Enzymes*, ed. Boyer, P. D., (Academic Press, New York), 3rd ed., Vol. III, pp. 81–118.
31. Eaton, W. A. & Hochstrasser, R. M. (1967) *J. Chem. Phys.* **46**, 2533–2536.
32. Margoliash, E., Smith, E. L., Kreil, G. & Tuppy, H. (1961) *Nature* **192**, 1125–1127.