Vik, S. B., Georgevich, G., & Capaldi, R. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1456–1460.
Wang, C.-S., & Smith, R. L. (1975) *Anal. Biochem. 63*, 414–417.
Wikstrom, M. (1984) *Nature (London) 308*, 558–560.
Wikstrom, M., & Saari, H. T. (1977) *Biochim. Biophys. Acta 462*, 347–361.
Yonetani, T. (1967) *Methods Enzymol. 10*, 332–336.

Variations in the Oxidation–Reduction Behavior of Liganded Species of *Pseudomonas* Cytochrome Oxidase†

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ABSTRACT: In an effort to determine the steady-state redox properties of *Pseudomonas aeruginosa* cytochrome cd_1, changes in absorption spectra after the addition of excess reductant (ascorbate, ferrous ethylenediaminetetraacetic acid) were monitored for degassed unliganded enzyme and samples in the presence of CO and CN^- at pH 6.0, 8.0, or 10.0. Plots of $[c^{2+}]/[c^{3+}]$ vs. $[d^{2+}]/[d^{3+}]$ indicate that a "pseudoequilibrium" was reached for all samples at pH 8.0. Calculated values of ΔE_{d-c}, the difference in reduction potential between the heme *c* and heme *d* moieties, at pH 8.0 were -25 ± 5 (unliganded), -10 ± 5 (enzyme–CO), and -25 ± 5 mV (enzyme–CN). Relative rates of heme *c* and heme *d* reduction were found to be dependent upon type of ligand, reductant, and pH. Evidence for a cooperative heme *c*–heme *d* interaction is discussed.

Pseudomonas aerguinosa cytochrome cd_1 (ferrocytochrome *c*-551:oxygen oxidoreductase, EC 1.9.3.2) is the terminal enzyme in the electron-transport chain of bacteria grown anaerobically in the presence of nitrate (Yamanaka et al., 1963). This water-soluble enzyme is a dimer of M_r 120 000; each subunit containing a heme *c* and a heme *d* (Kuronen & Ellfolk, 1972; Gudat et al., 1973; Kuronen et al., 1975; Parr et al., 1976; Saratse et al., 1977). Its physiological function is the one-electron reduction of NO_2^- to NO; however, the enzyme is also capable of catalyzing the four-electron reduction of O_2 to H_2O (Yamanaka et al., 1961), utilizing electrons donated either from *Pseudomonas* ferrocytochrome *c*-551 or reduced azurin. Electrons are donated to the enzyme at the heme *c* sites (Wharton et al., 1973; Parr et al., 1977), and substrate reduction occurs at heme *d* (Yamanaka & Okunuki, 1963a). While the former is covalently bound to the protein, the latter heme moiety is bound noncovalently and may be extracted and subsequently reinserted (Yamanaka & Okunuki, 1963b; Hill & Wharton, 1978). Therefore, those portions of the enzyme's absorption spectrum belonging to each heme type have been identified and found to be significantly separated, so that changes at the heme sites due to ligand binding and electron transfer may be easily monitored.

While extensive information is available on the rather complex kinetics of ligand binding and electron transfer within *Pseudomonas* cytochrome oxidase (Parr et al., 1975, 1977; Barber et al., 1978; Greenwood et al., 1978; Shickman & Gray, 1981), relatively little is known about the steady-state behavior of this enzyme. Previous investigators have carried out reductive titrations of the enzyme by using both excess (Shimada & Orii, 1976) and stoichiometric (Horio et al., 1961; Blatt & Pecht, 1979) amounts of reductant [ascorbate, ferrous ethylenediaminetetraacetic acid (FeEDTA), and/or durohydroquinone] and monitoring changes in the absorption spectrum with time. Using these two techniques has produced somewhat different results. Both Horio et al. (1961) and Blatt and Pecht (1979) present absorption spectra where the oxidized heme *d* peak at 640 nm shifts to ~625 nm upon reduction with no isosbestic points in the 600–700-nm region and calculate a positive difference in reduction potential between heme *c* and heme *d* (ΔE_{c-d}) of 60–70 mV. In the heme *d* absorption spectrum of Shimada and Orii (1976), the 640-nm peak shifts to 654 nm in the reduced spectrum, an isosbestic point appears at 650 nm, and ΔE_{c-d} is calculated to be 24 mV. Previously reported variations in the reduced heme *d* spectrum were only observed when dithionite was used as the reductant (Parr et al., 1974).

In an effort to resolve these discrepancies, we present the results of reductive titrations of *Pseudomonas* cytochrome oxidase under a variety of anaerobic conditions at room temperature: (1) Unliganded enzyme, pH 8.0, was examined with both excess and stoichiometric amounts of ascorbate and ferrous EDTA as reductants; (2) in the presence of excess ascorbate, the reduction process was examined at pH 6.0, 8.0, and 10.0 for the unliganded enzyme and samples to which either CO or CN^- had been added. Relative rates of heme *c* and heme *d* reduction have been found to vary under these conditions, differences in reduction potential of the two hemes occur as a function of ligand, and there is apparently cooperative interaction between heme *c* and heme *d*.

Experimental Procedures

Materials. Pseudomonas cytochrome oxidase was prepared according to the method of Gudat et al. (1973) and used without further purification. Enzyme concentration was 1–5 μM in titrated samples. Sodium ascorbate was prepared by neutralization of a saturated solution of ascorbic acid with 10

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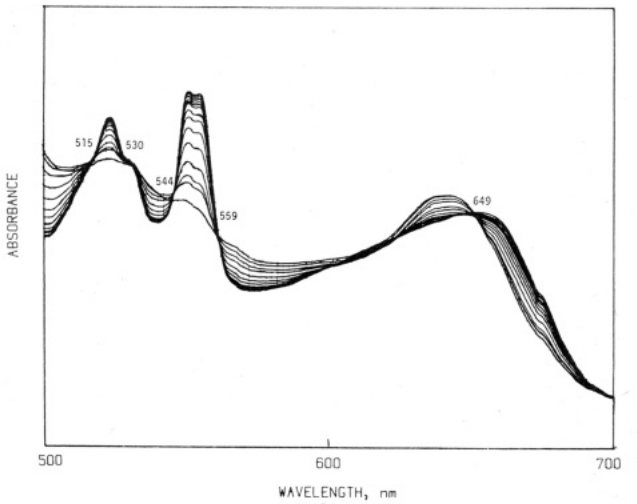


FIGURE 1: Anaerobic reduction of unliganded *Pseudomonas* cytochrome oxidase with excess solid ascorbate. Enzyme concentration was 1.18 μM in 0.1 M Tris buffer, pH 8.0. Scanning speed was 150 nm/min; successive scans were taken at 5-min intervals.

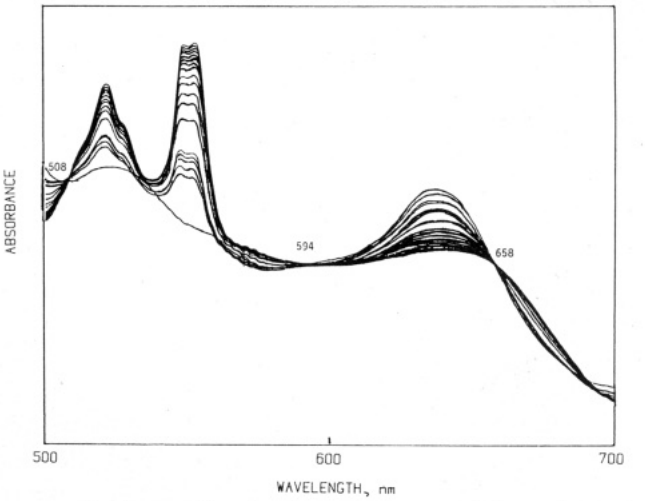


FIGURE 2: Anaerobic reduction with excess solid ascorbate of *Pseudomonas* cytochrome oxidase in 0.1 M Tris buffer, pH 8.0, saturated with CO. Enzyme concentration was 1.30 μM. Scanning conditions were the same as in Figure 1.

M NaOH. The solution was concentrated to half its original volume and cooled and cold ethanol added to precipitate the salt. The crystalline precipitate was then washed once with cold H_2O and twice with cold ethanol and used without further purification. Ferrous EDTA (10^{-4} M) was prepared in degassed 0.1 M Tris buffer, pH 8.0, according to the procedure of Blatt and Pecht (1979).

Reduction. All samples were prepared in 0.1 M Bis-Tris ([bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane), Tris [tris(hydroxymethyl)aminomethane], or CAPS (3-cyclohexylamino-1-propanesulfonic acid) buffer adjusted to the appropriate pH (6.0, 8.0, or 10.0) with 1 M HCl/NaOH. Samples were then placed in a 1-cm path-length anaerobic quartz cell equipped with a side arm for reductant addition. Samples were degassed by use of at least five cycles of evacuation until gas evolution ceased followed by saturation with N_2 that had been passed through a Lab Clear Model RGP-250 rechargeable gas purifier. Enzyme–CO and enzyme–CN samples were then prepared by saturation with CO or addition of excess 0.1 M KCN in sample buffer under a stream of N_2, as appropriate. If excess reductant was used, solid ascorbate or 10^{-4} M ferrous EDTA was added at the side arm under a stream of N_2 and the unit sealed prior to mixing of enzyme and reductant. Stoichiometric titrations were carried out by fitting a Hamilton Model TP 1001 gas-tight syringe containing either 10^{-4} M ascorbate or ferrous EDTA in 0.1 M buffer at the side arm. Absorption spectra were scanned at room temperature (23–25 °C) with a Perkin-Elmer Model 559A UV/vis spectrophotometer. Spectra were recorded prior to and within 30 s of reductant addition (first addition in the case of stoichiometric runs) and at 5-min intervals thereafter until two successive scans were identical. There was a lag time of 40 s between the scanning of the heme *d* and heme *c* spectral regions, which, on the basis of the total time for complete reduction (45–90 min) and the fact that a "pseudoequilibrium" state is reached in all reductions at pH 6.0 and 8.0 (see Results), is not deemed significant. At least three samples were run under each set of conditions cited above.

Results and Discussion

A reductive titration of unliganded enzyme at pH 8.0 with excess ascorbate is presented in Figure 1. Reduced heme *c* peaks occur at 522, 549, and 553 nm, and the 640-nm peak of oxidized heme *d* shifts to 654 nm. Isosbestic points at 515, 530, 544, 559, and 649 nm are in excellent agreement with the data of Shimada and Orii (1976) of 512, 530, 542, 558, and 650 nm, respectively. No variation in isosbestic points or the shape and position of absorption maxima was observed when a stoichiometric reduction was carried out under the same conditions; however, the protein tended to be unstable in its partially reduced state. In the absence of excess reductant, the protein precipitated from solution on approximately the same time scale required to perform a reductive titration (this situation was slightly mitigated in the presence of CN^-). As a result, complete reduction was difficult to achieve under stoichiometric conditions. Therefore, all spectral data presented here are the results of monitoring the reductive process in the presence of excess reductant.

In agreement with previous investigators (Shimada & Orii, 1976; Horio et al., 1961; Blatt & Pecht, 1979; Schichman & Gray, 1981), we note that heme *c* is more readily reduced than heme *d*. Reduction appears to be cooperative (Blatt & Pecht, 1979), and reduction of heme *d* is complete 5–10 min before that of heme *c*. However, while measurable amounts of reduced heme *d* are present within 30 s of ascorbate addition, as evidenced by the decrease in intensity of the oxidized heme *d* peak at 640 nm (see Figure 1), this is not the case when ferrous EDTA is the reductant. While the final absorption spectrum is identical with that of ascorbate-reduced enzyme, heme *c* is approximately 50% reduced before any change is observed in the 640-nm heme *d* peak, and heme *c* reduction is complete prior to that of heme *d*. In fact, we have observed that the reduced heme *c* absorption peaks reach a maximum, then slightly decrease in intensity as heme *d* reduction continues, and return to the maximum when reduction is complete. These results are in contrast to those of Blatt and Pecht (1979), who observed no difference in the reductive process when either ascorbate, ferrous EDTA, or durohydroquinone was used as the reductant. We are also unable to corroborate their finding that the fully reduced, unliganded protein exhibits a heme *d* absorption peak at ~625 nm. However, a heme *d* peak is present at ~620 nm for the fully reduced enzyme in the presence of CN^- (see below). We speculate that the data of these previous studies were obtained from liganded protein samples.

Spectral data for ascorbate-reduced CO–enzyme and CN–enzyme are shown in Figures 2 and 3, respectively. In Figure 2, the unliganded isosbestic point at 515 nm has shifted to 508

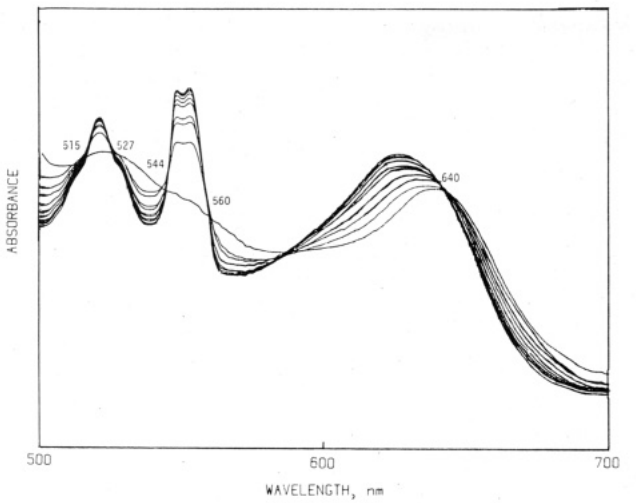


FIGURE 3: Anaerobic reduction with excess solid ascorbate of *Pseudomonas* cytochrome oxidase in 0.1 M Tris buffer, pH 8.0, in the presence of CN^-. Prior to reductant addition, 1 drop/mL of sample of 0.1 M KCN in sample buffer was added under a stream of N_2. Enzyme concentration was 1.32 μM. Scanning conditions were the same as in Figure 1.

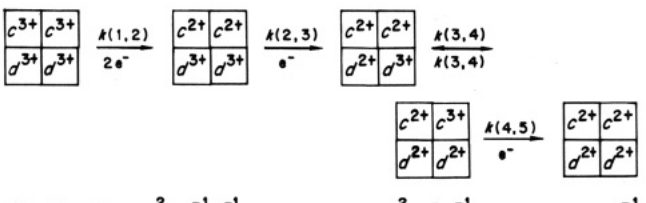
nm, 649 → 658 nm, and the remaining isosbestic points (530, 544, and 559 nm, Figure 1) are no longer present. As with ascorbate-reduced unliganded enzyme, heme *d* reduction is completed before that of heme *c*. While CO binds only to reduced heme *d* (Parr et al., 1975), Barber et al. (1978) have shown that CN^- slowly binds to both heme *c* and heme *d* when the enzyme is in its oxidized form but binds rapidly and exclusively to heme *d* in the reduced enzyme. Upon reduction of the oxidized CN–enzyme complex, heme *d* reduces immediately, whereas heme *c* reduction occurs over a period of several hours. The absorption spectra of Figure 3 are identical with data reported (Barber et al., 1978) for the titration of reduced enzyme with KCN. The 649-nm isobestic point shifts to 640 nm, as would be expected for a strong-field ligand bound to heme *d*, and the heme *c* spectral region is unchanged from that of the unliganded enzyme. We therefore conclude that within the time frame of our data collection cyanide binds only to the heme *d*, probably after reduction. In contrast to the unliganded enzyme and CO–enzyme ascorbate data, heme *c* reduction is complete approximately 5 min before that of heme *d*.

If, as has been proposed (Schichman & Gray, 1981), a steady-state that is in fact the rate-limiting step in the reduction process is reached during the course of electron transfer from heme *c* to heme *d*, one can then define (Shimada & Orii, 1976) a pseudoequilibrium constant:[1]

$$2c^{2+}d^{2+}d^{3+} \rightleftharpoons c^{2+}c^{3+}2d^{2+}$$

$$K = \frac{[c^{2+}][c^{3+}][d^{2+}]^2}{[c^{2+}]^2[d^{2+}][d^{3+}]} = \frac{[c^{3+}][d^{2+}]}{[c^{2+}][d^{3+}]}$$

[1] Our notation is based upon the reduction mechanism of Schichman and Gray (1981):



$k(1,2) = 1.72 \times 10^2\ M^{-1}\ s^{-1}$; $k(2,3) = 1.04 \times 10^2\ M^{-1}\ s^{-1}$; $k(3,4) = 0.25\ s^{-1}$

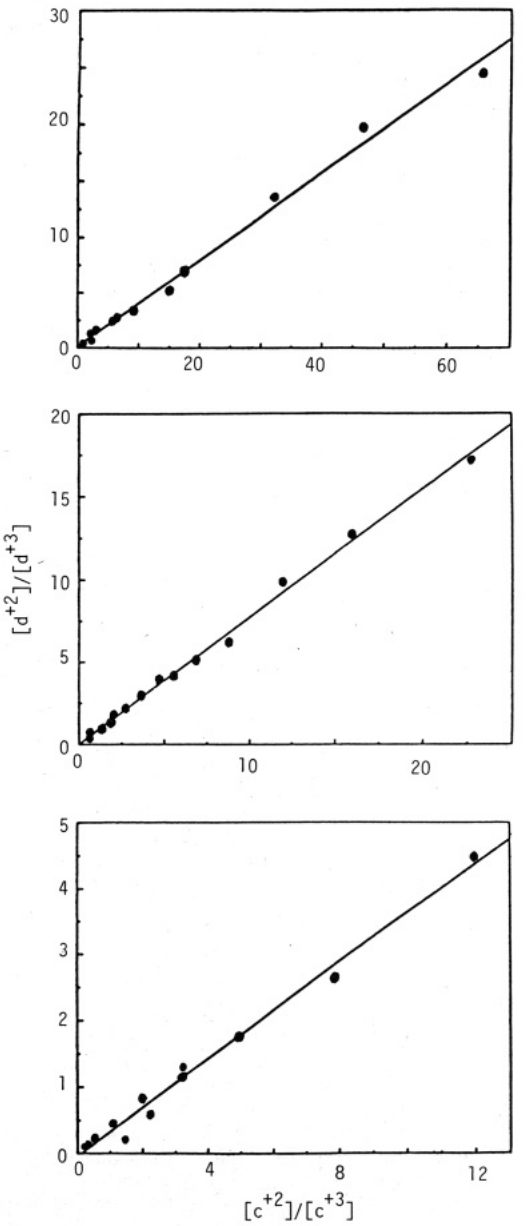


FIGURE 4: Plots of $[d^{2+}]/[d^{3+}]$ vs. $[c^{2+}]/[c^{3+}]$ for (top) enzyme–CN complex, (middle) enzyme–CO complex, and (bottom) unliganded enzyme. Each plot contains data from at least two spectral scans of the type in Figures 1–3. Correlation coefficients for each linear regression line are (top) 0.996, (middle) 0.998, and (bottom) 0.993.

If the postulated equilibrium is valid, plots of $[d^{2+}]/[d^{3+}]$ vs. $[c^{3+}]/[c^{2+}]$ from the data of Figures 1–3 should be straight lines passing through the origin. These results are shown in Figure 4, where each line represents data from at least two reductive titrations, with $[c^{3+}]/[c^{2+}]$ calculated at 553 nm and $[d^{2+}]/[d^{3+}]$ calculated at 640 nm for unliganded enzyme and CO–enzyme and at 625 nm for CN–enzyme. Since a steady-state approximation appears to be valid, the difference in reduction potential between the two hemes ΔE_{d-c} may be calculated:

$$\Delta E_{d-c} = 0.059 \log K$$

The value of ΔE_{d-c} for unliganded enzyme, -25 ± 5 mV, is identical with the results of Shimada and Orii (1976). An identical value of ΔE_{d-c} is obtained for the CN^--bound enzyme.

Conflicting results have been reported (Orii et al., 1977; Vickery et al., 1978; Blatt & Pecht, 1979) concerning the question of cooperative heme *d*–heme *c* interaction. We believe the reductive titration data for CO–enzyme in Figure 2 are

positive evidence for such an interaction. The value of ΔE_{d-c}, -10 ± 5 mV, is significantly lower than that of either the unliganded or CN-bound enzyme, but heme *c* is still more readily reduced than heme *d*. An analysis of the ligand binding kinetic data for CO predicts that, without heme *c*–heme *d* cooperativity, ligand binding would result in an increase of approximately 180 mV in the reduction potential of heme *d*.[2] The data presented here are not incontrovertible proof of redox cooperativity between the heme *c* and heme *d* sites. However, we believe that, in the absence of any indication of CO binding to either the heme *c* or oxidized heme *d* sites (this work; Parr et al., 1975; Tucker, 1982), some form of cooperativity between redox sites is strongly suggested.

Evidence for a change in the heme *c* reduction potential as a result of CO ligation at heme *d* is also provided by a Raman difference spectrum of the heme *c* vibrational modes of unliganded enzyme and CO–enzyme in which ν_4, the mode known to be sensitive to π-electron density (Spiro & Steakas, 1974), increases ~ 1.5 cm^{-1} in the liganded protein relative to the unliganded species (Y. C. Ching, D. L. Rousseau, M. R. Ondrias, and D. C. Wharton, unpublished results). This increase in ν_4 is indicative of a decrease in electron density in both the porphyrin π^* and iron d orbitals, and the heme *c* reduction potential would be expected to increase. Furthermore, although heme *c* is initially preferentially reduced, the fact that whether heme *c* or heme *d* reduction is complete first appears to be a function of both ligand and reductant not only supports the type of dynamic equilibrium suggested by Schichman and Gray (1981) but is also indicative of cooperative heme–heme interaction.

Reductive titrations with excess ascorbate were also carried out for all three enzyme species at pH 6.0 and 10.0. At pH 6.0, spectra were identical in appearance with those obtained at pH 8.0 and calculated ΔE_{d-c} values for unliganded and CO–enzyme were within experimental error of the values at the higher pH (−30 mV, unliganded; −5 mV, CO–enzyme). However, a "steady state" was never reached for CN–enzyme, indicating that the latter may have undergone a change in reaction kinetics as a result of the lower pH.

Although spectra at pH 10.0 also remained unchanged in appearance, both unliganded and CO–enzyme were completely reduced within 30 s of ascorbate addition. Heme *d* in CN–enzyme was also reduced immediately, whereas heme *c* reduction took ~ 100 min, indicating that CN^- had rapidly bound to both heme *c* and heme *d* (vide supra). Since $pK_a = 9.2$ for HCN, it is apparent that CN^- readily binds to both heme types, in contrast to the relatively slow binding of the protonated acid to the oxidized enzyme at pH <9.0. Given the above results, it was not possible to determine the effect of higher pH on the relative reduction potential of heme *c* and heme *d*.

In summary, we have demonstrated that reduction of heme *c* and heme *d* in *Pseudomonas* cytochrome oxidase is sensitive to changes in pH, ligand, and reductant. Steady-state kinetics were attained at pH 6–8 for both unliganded enzyme and CO–enzyme, making it possible to calculate the difference in reduction potential between the two hemes. Our results have also shown that using excess or stoichiometric amounts of reductant or changing pH has no effect upon the position of absorbance peaks or isosbestic points. However, the kinetics of reduction do vary with both pH and reductant, and Shichman and Gray (1981) have noted that the reaction kinetics are apparently more complex at low concentrations of reductant. Moreover, the protein tends to be unstable in its partially reduced form, making complete reduction difficult under stoichiometric conditions. These effects, coupled with variations in enzyme preparation and state of ligation, may be the source of apparent ambiguities in earlier results. Finally, our data for CO–enzyme provide reasonable evidence for cooperative heme *c*–heme *d* interactions.

Registry No. FeEDTA, 21393-59-9; CN^-, 57-12-5; CO, 630-08-0; cytochrome cd_1, 9027-00-3; ascorbic acid, 50-81-7; heme *c*, 26598-29-8; heme *d*, 60318-31-2.

[2] Since CO binds only to reduced heme *d*, at equilibrium

$$E^\circ_{+CO} = E^\circ_{-CO} + 0.59 \log (K_{assoc}[CO])$$

where $K_{assoc}[CO] > 1$. $K_{assoc} = k_{on}/k_{off} \sim 10^6$ (Parr et al., 1976); for a saturated solution at room temperature, $[CO] \sim 10^{-3}$ M.

REFERENCES

Barber, D., Parr, S. R., & Greenwood, C. (1978) *Biochem. J. 175*, 239.

Blatt, Y., & Pecht, I. (1979) *Biochemistry 18*, 2917.

Greenwood, C., Barber, D., Parr, S. R., Antonini, E., Brunori, M., & Colosimo, A. (1978) *Biochem. J. 173*, 11.

Gudat, J. C., Singh, J., & Wharton, D. C. (1973) *Biochim. Biophys. Acta 292*, 376.

Hill, K. E., & Wharton, D. C. (1978) *J. Biol. Chem. 253*, 489.

Horio, T., Kamen, M. D., & deKlerk, H. (1961) *J. Biol. Chem. 236*, 2783.

Kuronen, T., & Ellfolk, N. (1972) *Biochim. Biophys. Acta 275*, 308.

Kuronen, T., Saratse, M., & Ellfolk, N. (1975) *Biochim. Biophys. Acta 393*, 48.

Orii, Y., Shimada, H., Nozawa, T., & Masahiro, H. (1977) *Biochem. Biophys. Res. Commun. 76*, 983.

Parr, S. R., Wilson, M. T., & Greenwood, C. (1974) *Biochem. J. 139*, 273.

Parr, S. R., Wilson, M. T., & Greenwood, C. (1975) *Biochem. J. 151*, 51.

Parr, S. R., Barber, D., Greenwood, C., Phillips, B. W., & Melling, J. (1976) *Biochem. J. 157*, 423.

Parr, S. R., Barber, D., & Greenwood, C. (1977) *Biochem. J. 167*, 447.

Saratse, M., Virtanon, I., & Kuronen, T. (1977) *Biochim. Biophys. Acta 492*, 156.

Schichman, S. A., & Gray, H. G. (1981) *J. Am. Chem. Soc. 103*, 7794.

Shimada, H., & Orii, Y. (1976) *J. Biochem. (Tokyo) 80*, 135.

Spiro, T. G., & Strekas, T. C. (1974) *J. Am. Chem. Soc. 96*, 338.

Tucker, M. P. (1982) Ph.D. Thesis, Colorado State University.

Vickery, L. E., Palmer, G., & Wharton, D. C. (1978) *Biochem. Biophys. Res. Commun. 80*, 458.

Wharton, D. C., Gudat, J. C., & Gibson, Q. (1973) *Biochim. Biophys. Acta 292*, 611.

Yamanaka, T., & Okunuki, K. (1963a) *Biochim. Biophys. Acta 67*, 394.

Yamanaka, T., & Okunuki, K. (1963b) *Biochem. Z. 338*, 62.

Yamanaka, T., Ota, A., & Okunuki, K. (1961) *Biochim. Biophys. Acta 53*, 294.

Yamanaka, T., Kijimoto, S., & Okunuki, K. (1963) *J. Biochem. (Tokyo) 53*, 416.