THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 265, No. 20, Issue of July 15, pp. 11783-11787, 1990
Printed in U.S.A.

The Reaction of *Pseudomonas* Nitrite Reductase and Nitrite

A STOPPED-FLOW AND EPR STUDY*

(Received for publication, December 11, 1989)

Maria Chiara Silvestrini‡, Maria Gabriella Tordi, Giovanni Musci, and Maurizio Brunori§

From the ‡Departments of Biomedical Sciences and Biotechnologies and of Biometry, University of L'Aquila, L'Aquila 67100 and the Department of Biochemical Sciences and The Consiglio Nazionale delle Ricerche Center of Molecular Biology, University of Rome "La Sapienza," Rome, 00185 Italy

The reaction between reduced *Pseudomonas* nitrite reductase and nitrite has been studied by stopped-flow and rapid-freezing EPR spectroscopy. The interpretation of the kinetics at pH 8.0 is consistent with the following reaction mechanism (where k_1 and $k_3 \gg k_2$).

$$c^{2+}d_1^{2+} + NO_2^- \xrightarrow[(1)]{k_1} c^{2+}d_1^{3+}\cdot NO \xrightarrow[(2)]{k_2} c^{3+}d_1^{2+}\cdot NO \xrightarrow[e^-\ (3)]{k_3} c^{2+}d_1^{2+}\cdot NO$$

The bimolecular step (Step 1) is very fast, being lost in the dead time of a rapid mixing apparatus; the stoichiometry of the complex has been estimated to correspond to one NO_2^- molecule/heme d_1. The final species is the fully reduced enzyme with NO bound to heme d_1; and at all concentrations of nitrite, there is no evidence for dissociation of NO or for further reduction of NO to N_2O. Step 2 is assigned to an internal electron transfer from heme c to reduced NO-bound heme d_1 occurring with a rate constant of 1 s^{-1}; this rate is comparable to the rate of internal electron transfer previously determined when reducing the oxidized enzyme with azurin or cytochrome c_{551}. When heme d_1 is NO-bound, the rate at which heme c can accept electrons from ascorbate is remarkably increased as compared to the oxidized enzyme, suggesting an increase in the redox potential of the latter heme.

Pseudomonas cytochrome oxidase (ferrocytochrome c_{551}: oxygen oxidoreductase, EC 1.9.3.2) is a soluble redox enzyme involved in the electron transfer system of *Pseudomonas aeruginosa*. It is purified as a dimer of two identical noncovalently bound subunits of 60 kDa, each of which contains one heme c and one heme d_1 as prosthetic groups (1, 2). The enzyme was first identified (and thereafter referred to) by Horio (3) as an oxidase, able to reduce dioxygen to water using as electron donors homologous cytochrome c_{551} and azurin as well as a number of nonphysiological reductants. However, its physiological role, soon after discovered by Yamanaka *et al.* (4), is the reduction of nitrite in the denitrifying pathway of the bacterium. In fact, enzyme synthesis is induced when cells are grown in the presence of nitrate, and the turnover number for the oxidation of cytochrome c_{551} is higher when the electron acceptor is nitrite as compared to oxygen (5). Thus, *Pseudomonas* nitrite reductase, which was originally viewed as a simplified model for mitochondrial cytochrome oxidase (largely because of the presence of four prosthetic groups involved in the process of oxygen reduction), has now become a prototype for dissimilatory nitrite reductases, being the most studied among this class of enzymes.

In spite of the great deal of spectroscopic and kinetic information collected since its discovery (see, for instance, Refs. 6 and 7), several major problems are still unsolved. On one hand, structural information is still preliminary, and only very recently has the sequence of the enzyme been determined (8); on the other, the mechanism of the reaction with nitrite and the nature of the reaction product (NO or N_2O or both) are still largely obscure. In particular, rapid kinetic investigations on nitrite reduction are lacking. We report, in this paper, the results of a kinetic investigation aimed at shedding some light on presteady-state events occurring during nitrite reduction by cytochrome cd_1. The reaction is indeed very rapid, but only some of it is lost in the dead time of a stopped-flow apparatus; the new information obtained from these experiments together with rapid-freezing EPR spectroscopy allows us to draw some conclusions relevant to the mechanism of reaction with nitrite.

MATERIALS AND METHODS

Pseudomonas nitrite reductase was purified from *P. aeruginosa* cells (NCTC 6750) using the procedure of Parr *et al.* (9). The enzyme was electrophoretically and spectroscopically pure ($A_{410}/A_{280} > 1.1$; $A_{640}/A_{520} > 1.1$). Enzyme concentration was determined using, for the oxidized enzyme, the following extinction coefficient: ϵ_{412} = 282,000 M^{-1} cm^{-1} (10). Rapid kinetic experiments were carried out at 20 °C in 20 mM phosphate buffer (pH 8.0 or 6.2) using a Gibson-Durrum stopped-flow apparatus equipped with a 2-cm light path cell (dead time of 4 ms). Transient kinetics was followed at 460 nm, a wavelength typical of heme d_1, and at 425 and 550 nm, where mainly heme c is responsible for the spectroscopic signal (11).

Nitrite reductase, gently degassed and reduced with a 20-fold stoichiometric excess of sodium ascorbate, was mixed in the stopped-flow apparatus with nitrite at different concentrations under anaerobic conditions. The effect of the reducing power on the reaction time course was tested by increasing, in the enzyme-containing syringe, the concentration of ascorbate up to 1.2 mM in the absence or presence of cytochrome c_{551} (3-fold stoichiometric excess with respect to nitrite

* This work was supported by the Ministero dell'Università e della Ricerca Scientifica of Italy and by the Consiglio Nazionale delle Ricerche. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Dept. of Biochemical Sciences, University of Rome "La Sapienza," P. le A. Moro 5, Rome 00185, Italy.

reductase). Some experiments were carried out while mixing the reduced enzyme with nitrite solutions containing known concentrations of CO. To exclude rapid nonenzymatic production of NO by the ascorbate/nitrite system, hemoglobin (0.3 g/liter) reduced with 1 mM ascorbate was mixed in the stopped-flow apparatus with 1 mM nitrite, and the formation of the NO derivative was followed at 430 nm. Given the very high affinity of reduced hemoglobin for NO (K_d = 10^{-10} M (12)), any amount of NO is trapped by deoxyhemoglobin, and its production can be followed spectrophotometrically. This control shows that NO is indeed produced under these experimental conditions, but with a half-time of several minutes; thus, nonenzymatic artifacts in the time range of interest (seconds) are completely excluded.

EPR spectra were recorded at X-band on a Varian spectrometer equipped with a temperature control unit and interfaced to a Stelar Prometeus data system for acquisition and handling of the spectra. For EPR experiments, the reaction of the reduced enzyme with nitrite was quenched by rapidly injecting the outflow of a rapid-mixing homemade cell into an EPR quartz tube containing either isopentane chilled with liquid nitrogen or liquid nitrogen only; the dead time of this procedure was estimated to be ~100 ms. These experiments were carried out in 0.15M Tris-HCl (pH 8.0) to facilitate comparison with published EPR spectra (13).

Spectrophotometric measurements were carried out on a Cary 219 spectrophotometer.

RESULTS

It has been clearly demonstrated that NO binds readily and stoichiometrically to reduced heme d_1, at all pH values. Static spectrophotometric controls, obtained by incubating reduced nitrite reductase with nitrite in excess, gave results identical to those previously published (10, 13, 14). Some reaction with reduced heme c is also observed, but it is pH-dependent; and although slow, only at pH <7.0 is the rate of NO binding to heme c measurable (10, 14, 15). This pH-dependent behavior has been recently demonstrated by EPR and Mössbauer spectroscopy also for cytochrome *cd*$_1$ from *Thiobacillus denitrificans* (16); therefore, it seems characteristic of heme c present in this class of enzymes and at variance with eukaryotic ferrocytochrome *c* which binds NO only at very alkaline pH values (17). Since NO is the main (if not the only) catalytically significant reaction product (18–20), the kinetics of the reaction with nitrite was found to be simpler at alkaline pH values; thus, most of the measurements were carried out at pH 8.0, and the mechanistic considerations apply at this pH value.

Stopped-flow Experiments—Fig. 1 (*left panels*) shows the time course of the reaction observed at 460 and 425 nm upon mixing reduced nitrite reductase with excess nitrite at pH 8.0. At both wavelengths, the reaction is monophasic and can be described (within the errors) as a single exponential. The pseudo first-order rate constant (1 s^{-1}) is independent of observation wavelength and nitrite concentration up to 500 μM (after mixing). Thus, we are looking at the same monomolecular process at both wavelengths.

Stopped-flow experiments carried out in parallel at pH 6.2 show that the reaction is instead biphasic at all wavelengths, the fast phase being ~10 times faster than the only phase observed at pH 8.0, with an amplitude of opposite sign. Detailed analysis of the reaction kinetics has been carried out only at pH 8.0, and what follows refers to this condition.

The kinetic difference spectrum shown in Fig. 2 (*trace a*), which has its maximum around 450 nm, demonstrates that only heme d_1 is involved in the reaction. The amplitude of the absorbance change observed at 425 nm is perfectly compatible with the spectral contribution of heme d_1 at this wavelength (11). When the reaction was followed at 550 nm (a wavelength characteristic of heme c), an extremely small absorbance change was observed over the time range of the measurement (4 ms to 60 s) (see Fig. 3, *trace a*); thus, from

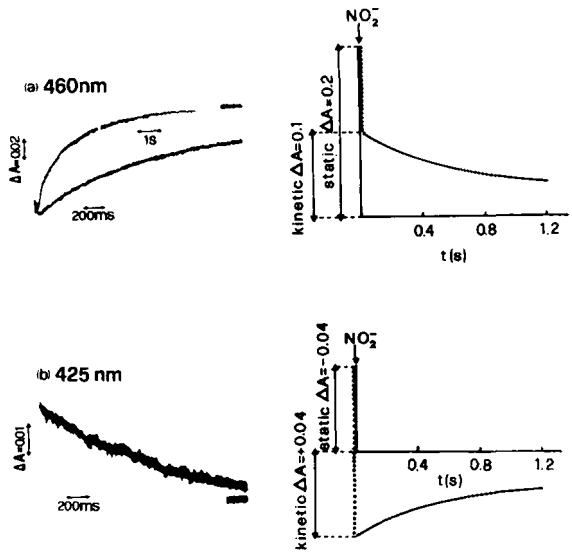


FIG. 1. **Transient kinetics of nitrite reduction by nitrite reductase.** *Left panels*, transmittance changes recorded at 460 nm (*a*) and 425 nm (*b*) upon mixing 5 μM nitrite reductase in the presence of 200 μM sodium ascorbate with 1 mM nitrite (concentrations before mixing). The buffer was 20 mM phosphate (pH 8.0), and the temperature was 20 °C. *Right panels*, the same traces are reported as absorbance changes and compared with the absorption change obtained by static spectrophotometry under identical conditions.

time = 4 ms to equilibrium, heme c is always more than 90% reduced.

The right panels of Fig. 1 and the difference spectra of Fig. 2 allow a comparison of the total absorbance change from static spectrophotometric measurements with that observed in the stopped-flow experiments. Such a comparison shows that a substantial fraction of the overall reaction is lost in the dead time of the instrument, even at the lowest concentrations of nitrite (which were substoichiometric). The rate of the process under observation is independent of substrate concentration; thus, the bimolecular reaction leading to the enzyme-nitrite complex and the subsequent redox reaction are lost in the dead time of the apparatus. On the assumption of second-order behavior, the minimum estimate for the rate constant for the formation of the E·S complex is $k_1 \geq 10^8\ M^{-1}\ s^{-1}$.

The transient observed is also independent of the concentration of the reductant, since, at fixed nitrite concentration, its rate does not change increasing the ascorbate concentration 20-fold (*i.e.* from 30 to 600 μM after mixing). Moreover, the time course is identical if the reaction with nitrite is carried out in the presence of CO (up to 0.5 mM after mixing).

Ferrocytochrome c_{551} added to the enzyme-containing syringe is oxidized stoichiometrically with heme c of the enzyme and with a rate constant of 1 s^{-1} (*i.e.* equal to the process observed on the enzyme in the absence of cytochrome c_{551} (Fig. 3, *trace b*)).

The dependence of the reaction amplitude at 460 nm on nitrite concentration (Fig. 4), established from kinetic data, allows the determination of a stoichiometry of one NO_2^- molecule/heme d_1. This finding is perfectly consistent with results obtained in equilibrium titration experiments of reduced nitrite reductase with nitrite (10) and demonstrates that the observed monomolecular process is coupled to formation of a 1:1 complex between heme d_1 and its substrate.

Rapid-freezing EPR Spectroscopy—To substantiate with independent evidence our interpretation of the stopped-flow data, rapid-quenching EPR experiments were carried out at pH 8.0. The EPR spectrum of the frozen (~120 K) mixture

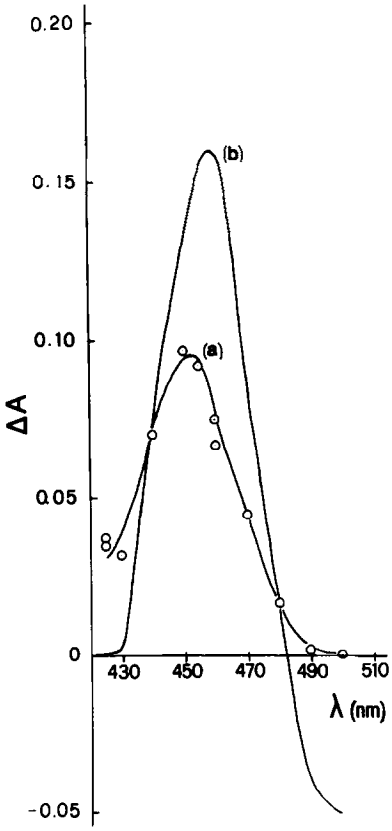


FIG. 2. **Kinetic and static difference spectra for reaction between reduced nitrite reductase and nitrite.** The kinetic difference spectrum (*trace a*) was calculated as the difference in absorbance between 4 ms and infinity in the stopped-flow apparatus (light path of 2 cm). Concentrations of nitrite reductase and nitrite were 2.5 and 250 μM, respectively (after mixing). The static difference spectrum (*trace b*), obtained under identical conditions, is shown for comparison. All other experimental conditions were the same as described for Fig. 1.

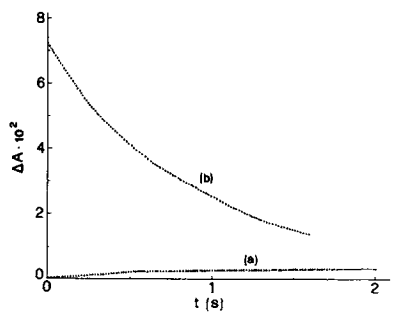


FIG. 3. **Reaction time course observed at 550 nm in the absence and presence of cytochrome c_{551} as electron donor.** *Trace a*, absorbance change observed at 550 nm upon mixing 3.6 μM nitrite reductase in the presence of 1 mM ascorbate with 1 mM nitrite; *trace b*, the same reaction after addition, to the enzyme-containing-syringe, of 27 μM ferrocytochrome c_{551}.

(aged ~50–100 ms) (Fig. 5, *trace a*) was compared with the equilibrium condition achieved by thawing and slowly refreezing the same solution. The spectrum of the equilibrium species (Fig. 5, *trace b*) is identical to that previously reported (13) for the fully reduced enzyme complexed with NO at heme d_1; this is borne out more clearly in Fig. 5 (*trace c*), which depicts the spectrum recorded at a lower modulation amplitude (0.25 millitesla) to determine the hyperfine splitting of the g_z component of the rhombic signal. Spin quantification of the equilibrium spectrum yields a value of 1.0 ± 0.2 spin/heme

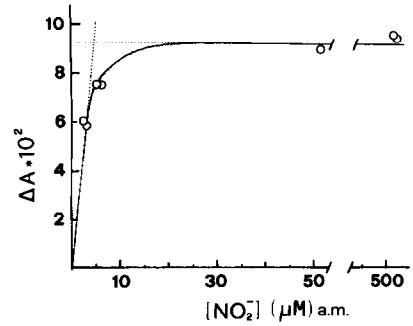


FIG. 4. **Dependence of reaction amplitude on nitrite concentration.** The absorbance changes observed at 460 nm upon mixing nitrite reductase with nitrite are reported as a function of nitrite concentration (after mixing). The enzyme concentration after mixing was 2.5 μM, corresponding to a d_1^{2+} concentration of 5 μM. All other experimental conditions were as described for Fig. 1.

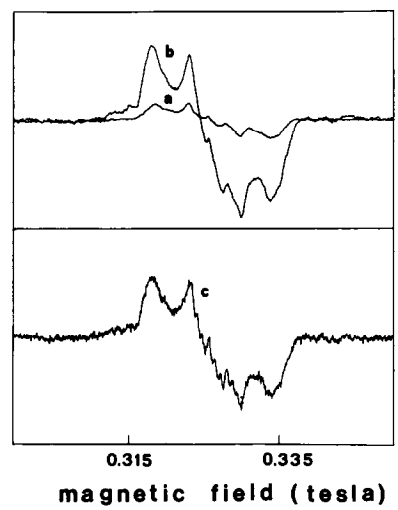


FIG. 5. **EPR spectra.** The spectra were recorded at X-band and 120 K. The microwave power was 2 milliwatts; the modulation amplitude was 0.8 millitesla (*traces a* and *b*) or 0.25 millitesla (*trace c*). The buffer was 0.15 M Tris-HCl (pH 8.0). *Trace a*, spectrum of the reaction mixture containing 47.5 μM nitrite reductase, 15 mM sodium ascorbate, and 500 μM nitrite (after mixing) frozen within 100 ms; *trace b*, spectrum of the reaction mixture in *trace a* after thawing and slow refreezing to attain equilibrium; *trace c*, *trace b* run at a lower modulation amplitude.

d_1, in accordance with previous observations (13, 16). Thus, we conclude that the species produced at infinite time in the stopped-flow apparatus is indeed the reduced enzyme with NO bound to heme d_1. Since the process observed by the stopped-flow experiments has a $t_{1/2}$ of 0.7 s, a quenched reaction mixture aged ~100 ms should mostly contain the transient and only a small percentage of the final product. As shown in Fig. 5 (*trace a*), the quenched mixture is characterized by an EPR spectrum identical in shape to that of the equilibrium state, but much smaller in intensity (*i.e.* ~20% of the total signal). This is consistent (within a factor of 2) with the concentration of the equilibrium species expected for a first-order reaction with $k = 1\ s^{-1}$ after 100 ms. In summary, the reaction between reduced nitrite reductase and nitrite rapidly produces an enzyme intermediate with a characteristic absorbance at the level of heme d_1 (as shown by difference spectroscopy), but EPR-silent at liquid nitrogen temperature; this intermediate decays with a rate constant of $1\ s^{-1}$, leading to the EPR-detectable complex $c^{2+}d_1^{2+}\cdot NO$.

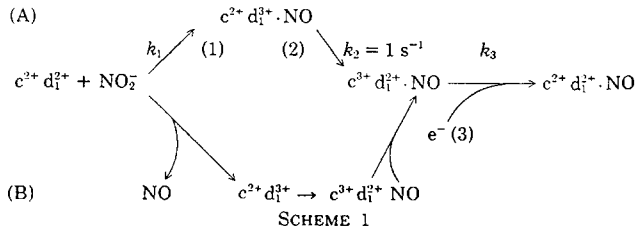
DISCUSSION

This is the first successful report of a rapid kinetic investigation on the reaction of nitrite with *Pseudomonas* nitrite reductase and provides novel information relevant to the mechanism of catalysis and to the interaction among the hemes. Scheme 1 summarizes the information obtained from the experiments at pH 8.0. The initial event in the reaction between the reduced enzyme and nitrite, although lost in the dead time of the stopped-flow apparatus, is shown to involve a bimolecular process with a 1:1 stoichiometry (Scheme 1, Step 1). Its occurrence can be inferred by comparison of the overall difference spectrum with that obtained for the observed transient (shown in Fig. 2). A minimum estimate for the relevant second-order rate constant associated with this initial event is $k_1 \geq 10^8$ M^{-1} s^{-1}. Comparison of this value with the second-order rate constant for the bimolecular reaction of the reduced enzyme with oxygen ($k = 3.3 \times 10^4$ M^{-1} s^{-1} (21)) reinforces the original proposal of Yamanaka *et al.* (4, 5) that the enzyme acts *in vivo* as a nitrite reductase.

The transient observed in the stopped-flow experiments, characterized by a rate constant of 1 s^{-1}, is assigned (Scheme 1, Step 2) to an intramolecular electron transfer from reduced heme c to the initial enzyme-substrate complex. This assignment is made on the basis of the following evidence: (i) The pseudo first-order rate constant of the process is independent of nitrite concentration; (ii) the species produced after rapid mixing has an absorption spectrum different from that of the reduced enzyme, but is EPR-silent at 120 K; and (iii) the species formed at equilibrium is identified by EPR spectroscopy as $c^{2+}d_1^{2+}\cdot NO$.

Observations at 425 and 550 nm show that heme c is more than 90% reduced at all times; thus, its re-reduction (Scheme 1, Step 3) should be much faster than the intramolecular electron transfer indicated as Step 2. Moreover, since the oxidation of the externally added ferrocytochrome c_{551} (which transfers electrons directly to heme c of nitrite reductase (22)) is synchronous with Step 2, the possibility that ascorbate directly reduces the $d_1^{3+}\cdot NO$ complex is excluded. It is known that the electron transfer rate from ascorbate to heme c of the oxidized enzyme is very low ($k = 1.7$ M^{-1} s^{-1} at pH 7.0 (23)). Even at pH 8.0, where this rate constant may increase by an order of magnitude because the electron donor is the ascorbate dianion (24), the rate of re-reduction may be insufficient to account for the observation that heme c is always >90% reduced. Thus, we postulate a substantial change in the parameters governing long-range electron transfer and possibly an increase in the redox potential of heme c upon binding of NO to heme d_1. This hypothesis is realistic in light of other data which indicate the existence of heme-heme interactions in nitrite reductase and a change in heme c redox potential upon binding of CO to heme d_1 (22).

The value of k_2 (1 s^{-1}) can be compared with independent estimates of the internal electron transfer from heme c to heme d_1, which (in the absence of external ligands of heme d_1) occurs with a first-order rate constant ranging from 0.2 to 2 s^{-1} (22, 25, 26). In the presence of oxygen, which binds at the reduced heme d_1 iron, the internal electron transfer is much faster and is undetermined ($k > 100$ s^{-1} (21)). Thus, whereas oxygen as a ligand of heme d_1 enhances the internal electron transfer rate, NO leads to no changes, at least at pH 8.0.



SCHEME 1

The species obtained in the stopped-flow experiments at infinite time is the fully reduced enzyme with NO bound to heme d_1, as demonstrated by: (i) static spectrophotometry (Refs. 10 and 13–16 and this work) and (ii) the EPR spectra at equilibrium (Fig. 5, *traces b* and *c*). This species may in principle be formed along two pathways (shown as A and B in Scheme 1) that differ in the nature of the intermediate produced by the rapid bimolecular reaction between NO_2^- and the reduced enzyme. Pathway A, just discussed, assumes that NO never dissociates from the active site, whereas pathway B assumes that NO dissociates after the rapid initial step and thereafter rebinds synchronously with the internal electron transfer. Although the two are difficult to discriminate between, pathway A is preferred, because in the presence of a large excess of CO (up to 0.5 mM after mixing), the time course is unaffected. In fact, at a CO/NO concentration ratio of 100, the free d_1^{2+} postulated in pathway B should be trapped by CO, with a pseudo first-order rate constant of ~10 s^{-1} (27), contrary to observation.

These results therefore indicate that under our conditions NO remains tightly bound to reduced heme d_1 and that only a fraction of reduced cytochrome c_{551} (stoichiometric with heme c of the enzyme) is oxidized in spite of the presence of a large excess of NO_2^-. This finding is perfectly in line with the limited capability of the enzyme to catalyze oxidation of reduced azurin by nitrite under steady-state conditions at pH 8.0 (10). The data recall our attention to the unsolved problem of the enzyme reaction mechanism under physiological conditions. At pH values <7.0, which are more similar to those prevailing in the periplasmic space, where the enzyme and its macromolecular substrates have been located (8, 28–30), this inhibition is apparently released (10); however, we do not know by which mechanism since even at this pH the binding constant of NO to heme d_1 remains very high and also heme c (in both oxidation states) becomes susceptible to complex formation with NO. More complete rapid kinetic experiments under physiological conditions seem thus particularly relevant and are presently underway.

Acknowledgments—We are indebted to Profs. Colin Greenwood, Michael Wilson, and Lilia Calabrese for helpful discussions.

REFERENCES

1. Kuronen, T., and Ellfolk, N. (1972) *Biochim. Biophys. Acta* **275,** 308–318
2. Gudat, J. C., Singh, J., and Wharton, D. C. (1973) *Biochem. Biophys. Res. Commun.* **292,** 376–390
3. Horio T. (1958) *J. Biochem. (Tokyo)* **45,** 267–279
4. Yamanaka, T., Ota A., and Okunuki, K. (1961) *Biochim. Biophys. Acta* **53,** 294–308
5. Yamanaka, T., and Okunuki, K. (1963) *Biochim. Biophys. Acta* **67,** 394–406
6. Henry, Y., and Bessières, P. (1984) *Biochimie (Paris)* **66,** 259–289
7. Brunori, M., Colosimo, A., Silvestrini, M. C., and Tordi, M. G. (1986) in *Frontiers in Bioinorganic Chemistry* (Xavier, A. V., ed) pp. 311–325, Verlagsgesellschaft, Weinheim
8. Silvestrini, M. C., Galeotti, C., Gervais, M., Schininà, E., Barra, D., Bossa, F., and Brunori, M. (1989) *FEBS Lett.* **254,** 33–38
9. Parr, S. R., Barber, D., Greenwood, C., Phillips, B. W., and Melling, J. (1979) *Biochem. J.* **157,** 423–430
10. Silvestrini, M. C., Colosimo, A., Brunori, M., Walsh, T. A., Barber, D., and Greenwood, C. (1979) *Biochem. J.* **183,** 701–709

11. Barber, D., Parr, S. R., and Greenwood, C. (1977) *Biochem. J.* **163,** 629–632
12. Antonini, E., and Brunori, M. (1971) *Hemoglobin and Myoglobin in Their Reactions with Ligands,* North-Holland Publishing Co., Amsterdam
13. Johnson, M. K., Thomson, A. J., Walsh, T. A., Barber, D., and Greenwood, C. (1980) *Biochem. J.* **189,** 285–294
14. Shimada, H., and Orii, Y. (1975) FEBS Lett. **54,** 237–240
15. Shimada, H., and Orii, Y. (1978) *J. Biochem. (Tokyo)* **84,** 1553–1558
16. Liu, M.-C., Huynh, B.-H., Payne, W. J., Peck, H. D., Dervartanian, D. V., and Legall, J. (1987) *Eur. J. Biochem.* **169,** 253–258
17. Ehrenberg, A., and Szczepkowski, T. W. (1960), *Acta Chem. Scand.* **14,** 1684–1692
18. Wharton, D. C., and Weintraub, S. T. (1980) Biochem. Biophys. Res. Commun. **97,** 236–242
19. Kim C. H., and Hollocher, T. C. (1983), *J. Biol. Chem.* **258,** 4861–4863
20. Shapleigh, J. P., Davies, K. J. P., and Payne, W. J. (1987), *Biochim. Biophys. Acta* **911,** 334–340
21. Greenwood, C., Barber, D., Parr, S. R., Antonini, E., Brunori, M., and Colosimo, A. (1978), *Biochem. J.* **173,** 11–17
22. Silvestrini, M. C., Tordi, M. G., Colosimo, A., Antonini, E., and Brunori, M. (1982), *Biochem. J.* **203,** 445–451
23. Barber, D., Parr, S. R., and Greenwood, C. (1978), *Biochem. J.* **175,** 239–249
24. Al-Ayash, A. I., and Wilson, M. T. (1979), *Biochem. J.* **177,** 641–648
25. Parr, S. R., Barber, D., Greenwood, C., and Brunori, M., (1977) *Biochem. J.* **167,** 447–455
26. Wharton, D. C., Gudat, J. C., and Gibson, Q. H. (1973), *Biochim. Biophys. Acta* **292,** 611–620
27. Parr, S. R., Wilson, M. T., and Greenwood, C. (1975), *Biochem. J.* **151,** 51–59
28. Wood, P. M. (1978), *FEBS Lett.* **92,** 214–218
29. Canters, G. W. (1987) *FEBS Lett.* **212,** 168–172
30. Nordling, M., Young, S., Karlsson, B. G., and Lundberg, L. G. (1990) *FEBS Lett.* **259,** 230–232