Proc. Nat. Acad. Sci. USA
Vol. 71, No. 1, pp. 140–143, January 1974

Proton Magnetic Resonance and Magnetic Susceptibility Characterization of Ferredoxin I from *Bacillus polymyxa*

(iron-sulfur protein/bacterial ferredoxins/contact-shifted resonances)

WILLIAM D. PHILLIPS*, C. C. McDONALD*, N. A. STOMBAUGH†, AND W. H. ORME-JOHNSON†

* E. I. du Pont de Nemours and Company, Central Research Department, Experimental Station, Wilmington, Delaware 19898; and
† Department of Biochemistry, University of Wisconsin, Madison, Wisc. 53706

Contributed by William D. Phillips, September 7, 1973

ABSTRACT **Magnetic susceptibility and proton magnetic resonance spectra are reported for the oxidized and reduced forms of the iron-sulfur protein *Bacillus polymyxa* ferredoxin I. The magnetic susceptibility of the oxidized form indicates antiferromagnetic exchange coupling between component iron atoms that is quantitatively similar to that observed for the clostridial ferredoxins and for the $[(C_2H_5)_4N]_2[Fe_4S_4(SCH_2C_6H_5)_4]$ analog. Contact-shifted resonances observed in the proton-magnetic-resonance spectra of oxidized and reduced forms of the *B. polymyxa* protein can be correlated with the contact-shifted resonances of corresponding redox forms of the clostridial ferredoxins. Characteristics of the contact-shifted resonances observed in partially reduced *B. polymyxa* ferredoxin I are compatible with a "slow" rate of electron exchange between redox forms, which suggests that the "fast" electron exchange earlier observed in the eight-iron clostridial ferredoxins may derive from an intramolecular component.**

In recent years, a new subdivision of bacterial ferredoxins containing only a single cluster of four iron and four labile sulfur atoms per molecule has become known; two members of this new subclass of bacterial ferredoxins have been isolated (1) from the facultative nitrogen fixing bacterium *Bacillus polymyxa* and their chemical, physical, and electron paramagnetic resonance (EPR) properties defined (1–4). Both of these proteins have molecular weights of about 9000 daltons and contain four iron atoms and four labile sulfur atoms per molecule. Both optical absorption and EPR spectra strongly suggest that the iron and labile sulfur atoms in the four-iron ferredoxin are arranged into a single tetrameric cluster similar to the two tetrameric clusters found in the more complex eight-iron bacterial ferredoxins (5). The purpose of this study was to investigate the magnetic susceptibility and proton magnetic resonance (PMR) properties of one of these four-iron ferredoxins, *B. polymyxa* ferredoxin I, in order to further characterize this new type of iron-sulfur protein. It was hoped also that the study of this simpler form of bacterial iron-sulfur protein would lead to a better understanding of PMR data collected on the eight-iron proteins.

The magnetic susceptibility and PMR results for both oxidized and reduced *B. polymyxa* ferredoxin I further emphasize the close electronic relationship between the Fe_4S_4 clusters of the bacterial ferredoxins and the high potential iron proteins of photosynthetic bacteria. It is reasonable to hypothesize from these and earlier findings that the Fe_4S_4 clusters are of the same basic geometrical type, a hypothesis that must necessarily be tested by crystallographic means and which emphasizes the role of the protein moiety in dictating the electron-transfer properties (potentials and specificities) of these proteins.

Abbreviations: PMR, proton magnetic resonance; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; BM, Bohr magnetons; χ_M^P, the paramagnetic component of the molar magnetic susceptibility; DSS, sodium 2,2-dimethyl-2-silapentanesulfonate; $\mu_{eff}^{22°}$, 22° value for the effective magnetic moments per iron atom expressed as units of the Bohr magneton.

MATERIALS AND METHODS

The *B. polymyxa* ferredoxin I employed in these studies was isolated and purified by methods previously described (1). Reductions were carried out using British Drug House $Na_2S_2O_4$. Nuclear magnetic resonance (NMR) spectra for the most part were obtained on a Varian 220 MHz PMR spectrometer; searches to extreme low-field were carried out on a Bruker 90 MHz spectrometer. Magnetic susceptibilities were determined by previously described NMR techniques (6).

RESULTS AND DISCUSSION

Magnetic Susceptibilities. Magnetic susceptibilities have been obtained previously in solution for the eight-iron ferredoxins derived from *Clostridium pasteurianum* (7) and *Clostridium acidi-urici* (8) and in the solid state for the synthetic analog of the bacterial ferredoxins $[(C_2H_5)_4N]_2[Fe_4S_4(SCH_2C_6H_5)_4]$ ref. (9). These previously observed magnetic susceptibilities, as well as PMR studies on the oxidized forms of the bacterial ferredoxins, strongly indicated that the component iron atoms are coupled by an antiferromagnetic exchange interaction. Principal evidence for this conclusion was the increase in magnitude of contact-shifted resonances with increasing temperature and the low values of the effective magnetic moments of the component iron. For example, as shown in Table 1, the 22° values for the effective magnetic moments per iron atom ($\mu_{eff}^{22°}$) for the oxidized forms of the ferredoxins derived from *C. pasteurianum* and *C. acidi-urici* are, respectively, 1.06 and 1.24 BM (Bohr magnetons). These values are to be contrasted with the moment of 5.85 BM obtained for the oxidized form of the rubredoxin from *C. pasteurianum* (10). This latter value is characteristic of high-spin ferric iron unperturbed by exchange interaction.

This conclusion concerning the presence of exchange interaction between component iron atoms of the eight-iron ferredoxins was reinforced by the recent extensive physical characterization of the synthetic iron-sulfur analog $[(C_2H_5)_4N]_2[Fe_4S_4(SCH_2C_6H_5)_4]$ (9). X-ray studies have shown that the Fe_4S_4 clusters of the reduced form of the high potential iron protein from *Chromatium* strain D (11), from the oxidized form of the eight-iron ferredoxin from *Peptococcus aerogenes* (5), and from $[Fe_4S_4(SCH_2C_6H_5)_4]^{=}$ are very closely related structurally (9, 12). Magnetic susceptibility studies of the dianion, in the solid state and to liquid helium temperatures, established the existence of antiferromagnetic exchange coupling

TABLE 1. $\mu_{eff}^{22°}$ *for three ferredoxins and a synthetic analog*

	$\mu_{eff}^{22°}$
Bacillus polymyxa ferredoxin	0.90
Clostridium pasteurianum ferrodoxin	1.06*
Clostridium acidi-urici ferrodoxin	1.24†
$[Fe_4S_4(SCH_2C_6H_5)_4]^=$	1.04‡

The values for $\mu_{eff}^{22°}$ refer to the oxidized forms of the ferredoxins and to the dianion form of the analog, and are expressed as units of the Bohr magnetons and calculated per iron atom under the assumption of equivalence of component iron atoms.

* Ref. 7. † Ref. 8. ‡ Ref. 9.

between component iron atoms of the Fe_4S_4 cluster. The magnitude for $\mu_{eff}^{22°}$ of 1.04 BM for the dianion is seen (Table 1) to be similar to values at corresponding temperatures for the two eight-iron bacterial ferredoxins and close to the value of 0.90 BM obtained for the oxidized form of the four-iron *B. polymyxa* ferredoxin I. Susceptibility studies thus indicate a magnetic strueture for the *B. polymyxa* ferredoxin I related to that possessed by the two clostridial ferredoxins and the well-characterized $[Fe_4S_4(SCH_2C_6H_5)_4]^=$ analog.

Magnetic susceptibilities were obtained for the reduced form of ferredoxin I from *B. polymyxa* in solution (Table 2), along with more extensive susceptibility measurements of the oxidized form. Results of susceptibility studies of the reduced forms of the ferredoxins must be viewed with more reserve than results for the oxidized forms because of the greater instabilities of the reduced form to decomposition of the Fe_4S_4 clusters, particularly in solution and at about room temperature. Precautions were taken, however, including reversibility checks of the susceptibilities between the two redox states, and it is felt that the results given in Table 2 for the reduced form are reasonably accurate. Decomposition of Fe_4S_4 clusters releases iron to the solution, and the contribution of the released iron to the susceptibility generally will be greater than when the iron is a component of an intact, exchange-coupled cluster of a protein molecule. To the extent that the listed values are in error, they are too large.

With this caveat in mind, it is seen that the magnetic susceptibilities and effective moments per iron atom are considerably larger for the reduced form of *B. polymyxa* ferredoxin I than values at corresponding temperatures for the oxidized form. In addition, although the temperature range over which these studies were conducted is quite narrow, it would appear that χ_M^P (the paramagnetic component of the molar magnetic susceptibility) for the reduced form is decreasing with increasing temperature. The susceptibility results for reduced *B. polymyxa* ferredoxin I can be treated in a number of ways. For example, if it is assumed that the electron added to form the reduced ferredoxin contributes the spin-only value of 1.73 BM to the over-all magnetic moment, it is easily seen from the results of Table 2 that each of the four iron atoms, assuming them to be equivalent, contributes an additional 1.4 BM to the total moment. This additional contribution of 1.4 BM presumably is determined by the magnitude of the antiferromagnetic exchange interaction that persists in the reduced form of the ferredoxin. The value of 1.4 BM calculated for the reduced form is greater than the 0.90 BM observed for the oxidized form and could reflect a reduction of the magnitude of the coupling parameter in the reduced

TABLE 2. *Paramagnetic susceptibility of Bacillus polymyxa ferredoxin I*

	Oxidized form		Reduced form	
Temperature	$10^3\,\chi_M^P$*	μ_{eff}†	$10^3\,\chi_M^P$*	μ_{eff}†
3°			5.0	1.7
5°	1.3	0.86		
15°	1.3	0.88	4.4	1.6
22°	1.4	0.90	4.4	1.6
30°	1.4	0.91		

* The paramagnetic component of the molar magnetic susceptibility is expressed in units of $cm^3\ mol^{-1}$.

† The effective magnetic moment per iron atom is expressed in units of the Bohr magneton.

ferredoxin. Such a reduction in the exchange interaction on proceeding from the oxidized to reduced state has been indicated from magnetic susceptibility studies of the two-iron plant ferredoxin from spinach (13).

PMR Studies. PMR spectra of the oxidized and reduced forms of the ferredoxin I from *B. polymyxa* have been obtained in solution for the oxidized form over the range 4°–69° and for the reduced form over the range 4°–50°. Spectra for these two forms of the ferredoxin obtained at 30° are shown in Figs. 1 and 2. Both spectra display the usual strong absorption in the 0–10 ppm range that are characteristic of the amino-acid composition of the protein and the way in which the protein folds in solution. Note that the spectra of the protein in this region of resonance absorption are quite different for the two forms. Both conformational differences and magnetic perturbations presumably contribute to the differences observed. Detailed analysis of this region of resonance absorption of the two redox forms of *B. polymyxa* ferredoxin I will be dealt with in a subsequent publication. Here, we are concerned with general features of resonances that have characteristics clearly dominated by contact interaction with component paramagnetic centers.

Resonances are observed to low-field of 10 ppm in the PMR spectra of *B. polymyxa* ferredoxin I which have breadth, position, and temperature dependences that clearly indicate the resonances to be subject to contact interaction. In oxidized *B. polymyxa* ferredoxin I, seven such resonances, each of an intensity corresponding to one proton per molecule of ferredoxin, are readily identified. The temperature dependence of these resonances are shown in the left-hand portion of Fig. 3. These resonances appear in the same general region of resonance absorption as do resonances with similar widths and temperature dependences that are observed for the oxidized forms of the ferredoxins from *C. pasteurianum* (7) and *C. acidi-urici* (8) and, also for resonances that derive from the reduced form of the high potential iron protein from *Chromatium* D (14). Predictably, fewer such resonances are observed for the *B. polymyxa* ferredoxin I than from the eight-iron bacterial ferredoxins. Earlier, these resonances for the oxidized clostridial ferredoxins were assigned, tentatively, to the β-CH_2 protons of the cysteinyl groups that were presumed to be responsible for attachment of the iron-sulfur clusters to the polypeptide chain. PMR studies of the $[Fe_4S_4(SCH_2C_6H_5)_4]^=$ analog and others of the series (15, 16), as well as x-ray crystallographic studies of the analog (9), the ferredoxin

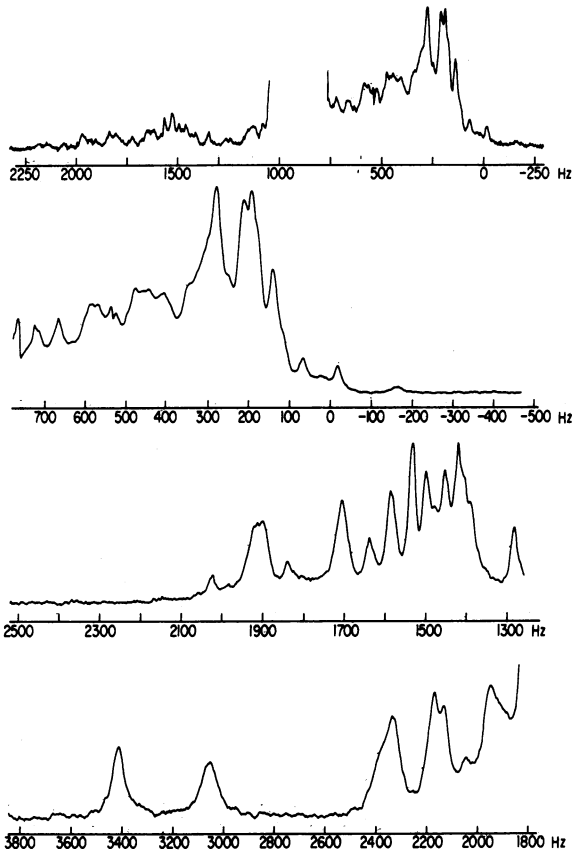

FIG. 1. PMR spectrum (220 MHz) of the oxidized form of *Bacillus polymyxa* ferredoxin I at 30°. The top spectrum is a single scan of the −250 Hz to 2250 Hz region of resonance absorption and does not extend sufficiently far to low-field to include the contact-shifted resonances displayed in the lower trace. The two middle displays are computer-averaged expansions of the saturated CH and aromatic CH regions, respectively, of proton resonance absorption. The bottom display is a computer averaged spectrum of the contact-shifted resonances, which almost certainly arise from the β-CH_2 protons of the four component cysteine residues. Protein concentrations were 59 mg/ml in D_2O and the pD was 7.0. All spectra were internally referenced to DSS.

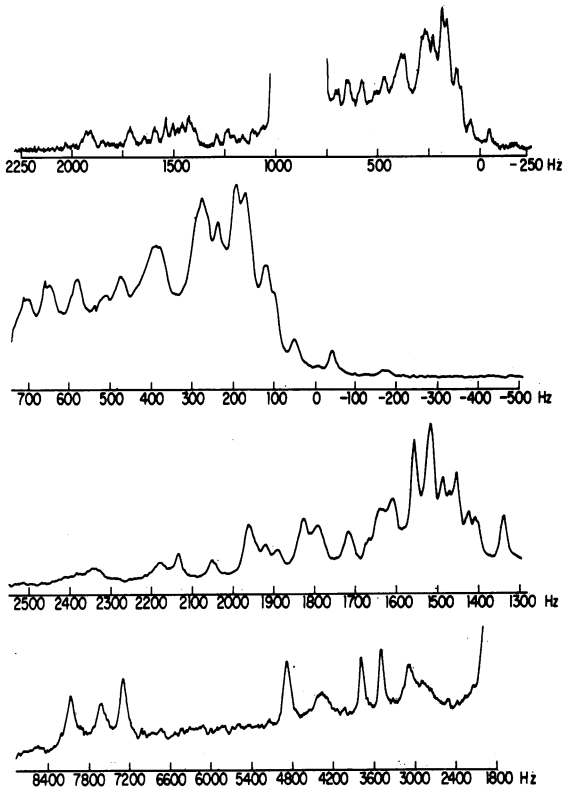

FIG. 2. PMR spectrum (220 MHz) of the reduced form of *Bacillus polymyxa* ferredoxin I at 30°. The top spectrum is a single scan of the −250 Hz to 2250 Hz region of resonance absorption and does not extend sufficiently far to low-field to include the contact-shifted resonances displayed in the lower trace. The two middle displays are computer-averaged expansions of the saturated CH and aromatic CH regions, respectively, of proton resonance absorption. The bottom display is a computer averaged spectrum of the contact-shifted resonances which are assigned to the β-CH_2 and α-CH protons of the four cysteine residues as well, possibly, as to other protons located near the paramagnetic center. Protein concentrations were 59 mg/ml in D_2O and the pD was 7.0. All spectra were internally referenced to DSS.

from *P. aerogenes* (5), and the high potential iron protein from *chromatium* D (11), largely dispel any doubts about this assignment to the β-CH_2 protons, at least as far as the oxidized forms are concerned. The general features of the contact-shifted resonances of the oxidized form of *B. polymyxa* ferredoxin I indicate that geometrically, electronically, and magnetically the iron-sulfur cluster is similar to the iron-sulfur clusters of the oxidized forms of the clostridial ferredoxins and the reduced form of the high potential iron protein from *Chromatium* D.

The temperature dependences of readily identifiable contact-shifted resonances of reduced *B. polymyxa* ferredoxin I are shown in the right-hand portion of Fig. 3. Again we note fewer contact-shifted resonances for reduced *B. polymyxa* ferredoxin I than for the reduced forms of the clostridial ferredoxins (7, 8), which is compatible with the existence of one rather than two Fe_4S_4 clusters in the *B. polymyxa* ferredoxin. Assignment of observed contact-shifted resonances in reduced *B. polymyxa* ferredoxin I is somewhat less certain than for the oxidized form. Certainly the positions of the β-CH_2 protons of the cysteinyl residues involved in binding the presumed Fe_4S_4 cluster are dominated by contact-interaction perturbation. The α-CH protons also are subject to a similar perturbation with an attenuation factor, as compared to β-CH_2 protons, of about 7.2. This factor was established from PMR studies of the synthetic dianion analogs (15, 16). A total of 12 contact-shifted resonances are therefore expected from the four cysteinyl residues of *B. polymyxa* ferredoxin I that presumably bind an Fe_4S_4 cluster to the polypeptide chain. Resonances that correspond to 11 protons are readily identified in the contact-shifted spectrum of the reduced protein. Detailed assignment of these resonances of β-CH_2 and to α-CH protons of individual cystienyl residues is not possible. The question arises as to whether additional contact-shifted resonances for the reduced form of *B. polymyxa* ferredoxin I occur even further to low-field than those plotted in Fig. 3. An unequivocal answer cannot, of course, be given. The 220 MHz PMR spectrometer available to the authors was capable of sweeps to only 9500 Hz (43 ppm) to low-field of the reference, sodium 2,2-dimethyl-2-silapentanesulfonate (DSS). The extreme low-field resonance of reduced *B. polymyxa* ferredoxin I

plotted in Fig. 3 (intensity corresponding to two protons) was in fact not observed in our 220 MHz PMR studies (Fig. 2). The resonance was detected in supplementary studies employing a Bruker 90 MHz PMR spectrometer that permitted sweeps to 88 ppm (equivalent to 19,400 Hz at 220 MHz) to low-field of the reference DSS. Thus, we can conclude that if there are additional contact-shifted resonances in the PMR spectrum of reduced *B. polymyxa* ferredoxin I, they are very far removed from anything heretofore detected. We consider this possibility unlikely.

In any event, the temperature dependence of the contact-shifted resonances of the reduced form of *B. polymyxa* ferredoxin I bear striking qualitative resemblances to those of the reduced forms of the clostridial ferredoxins. Unlike the oxidized form, there is no uniformity in the temperature dependence of the reduced form: some increase with temperature, some decrease with temperature, and some are almost temperature invariant. This variation in temperature dependence of contact shifts has been ascribed qualitatively to a nonuniform distribution of spin density in the reduced form of the protein so that the β-CH_2 and α-CH protons of the four iron-binding cysteinyl groups are experiencing contact interaction perturbations from this nonuniform spin density distribution (14). Such a nonuniform spin density distribution of the Fe_4S_4 cluster could result from perturbations of side chains such as aromatic rings near one or more surfaces of the cube. Such interactions have been postulated from ^{13}C-NMR studies of *C. acidi-urici* ferredoxin (17).

Monotonic variations in the positions of the contact-shifted resonances of the oxidized form of *B. polymyxa* ferredoxin I were observed over the range 4°–69° (Fig. 3). In addition, except for acceleration of deuterium exchange of some slowly exchanging amide NH protons, the rest of the PMR spectrum of the protein was largely unaffected by temperature elevation to 69°. It was clear that thermal denaturation of whatever folded conformation oxidized *B. polymyca* ferredoxin I possesses was not occurring during elevation of the temperature to 69°. Upon standing at 69°, however, the contact-shifted resonances were noted to decrease uniformly in intensity with a half-life of about 45 min, and other regions of the spectrum assumed characteristics that clearly reflect the loss of tertiary structure. The loss of tertiary structure progressed with the same half-life. Thus, *B. polymyxa* ferredoxin I, like *C. pasteurianum* ferredoxin, is not subject to thermal denaturation at fairly high temperatures (16). The Fe_4S_4 cluster here, as in *C. pasteurianum* ferredoxin, apparently plays a prominent role in the maintenance of tertiary structure.

An additional feature of the PMR study of *B. polymyxa* ferredoxin I is worth noting. Upon addition of the appropriate amount of $Na_2S_2O_4$ to the oxidized form, a solution consisting of approximately equal amounts of the two redox forms can be prepared. The resulting PMR spectrum consists of a simple superposition of the PMR spectra of the separate oxidized and reduced ferredoxins. Electron exchange between oxidized and reduced forms here is "slow" on the PMR time scale, in contrast to "fast" electron exchange between oxidized and reduced forms of *C. pasteurianum* ferredoxin in solutions prepared in similar fashion. "Slow" electron exchange similarly is observed between oxidized and reduced forms in solutions of the high-potential-iron protein from *Chromatium* D. It is suggestive, but far from proven at this point, that the apparently greater electron exchange rate between Fe_4S_4 clusters in the eight-iron clostridial ferredoxin derives at least partially from an intramolecular exchange mechanism.

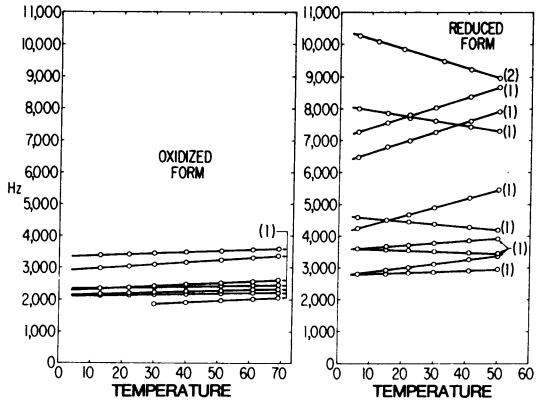

FIG. 3. Temperature dependence of identified contact-shifted resonances in the 220 mHz PMR spectra of the oxidized (*left*) and reduced (*right*) forms of the ferredoxin I from *Bacillus polymyxa*. Figures in parentheses denote numbers of protons per ferredoxin molecule that contribute to the resonance.

We thank Mr. F. V. Ferrari for excellent technical assistance in obtaining the reported PMR and magnetic susceptibility results. This work was supported in part by the National Institutes of Health (Research Grants AI 00848 and GM 17170, and Training Grant GM 236) and by the National Science Foundation (Grant GB-483). This paper is Contribution no. 2078 from the Central Research Department, E. I. du Pont de Nemours and Co., Wilmington, Del. 19898.

1. Stombaugh, N. A., Burris, R. H. & Orme-Johnson, W. H. (1973) *J. Biol. Chem.*, **248**, 7951–7956.
2. Shethna, Y. I., Stombaugh, N. A. & Burris, R. H. (1971) *Biochem. Biophys. Res. Commun.* **42**, 1108–1116.
3. Orme-Johnson, W. H., Stombaugh, N. A. & Burris, R. H. (1972) *Fed. Proc.* **31**, Abstr. 448.
4. Yoch, D. C. & Valentine, R. C. (1972) *J. Bacteriol.* **110**, 1211–1213.
5. Sieker, L. C., Adman, E. & Jensen, L. H. (1972) *Nature* **235**, 40–42.
6. Phillips, W. D. & Poe, M. (1972) in *Methods in Enzymology*, ed. A. SanPietro (Academic Press, New York), Vol. 24, 304–317.
7. Poe, M., Phillips, W. D., McDonald, C. C. & Lovenberg, W. (1970) *Proc. Nat. Acad. Sci. USA* **65**, 797–804.
8. Poe, M., Phillips, W. D., McDonald, C. C. & Orme-Johnson, W. H. (1971) *Biochem. Biophys. Res. Commun.* **42**, 705–713.
9. Herskovitz, T., Averill, B. A., Holm, R. H., Ibers, J. A., Phillips, W. D. & Weiher, J. F. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2437–2441.
10. Phillips, W. D., Poe, M., Weiher, J. F., McDonald, C. C. & Lovenberg, W. (1970) *Nature* **227**, 574–577.
11. Carter, C. W., Freer, S. T., Xuong, N. H., Alden, R. A. & Kraut, J. (1971) *Cold Spring Harbor Symp. Quant. Biol.* **36**, 381–385.
12. Carter, C. W., Kraut, J., Freer, S. T., Alden, R. A., Sieker, L. C., Adman, E. & Jensen, L. H. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3526–3529.
13. Palmer, G., Dunham, W. R., Fee, J. A., Sands, R. H., Iizuka, T. & Yonetani, T. (1971) *Biochim. Biophys. Acta* **245**, 201–207.
14. Phillips, W. D., Poe, M., McDonald, C. C. & Bartsch, R. G. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 682–687.
15. Holm, R. H., Phillips, W. D., Averill, B. A., Mayerle, J. J. & Herskovitz, T., *J. Am. Chem. Soc.*, in press.
16. McDonald, C. C., Phillips, W. D., Lovenberg, W. & Holm, R. H. (1973) *N. Y. Acad. Sci.*, in press.
17. Packer, E. L., Sternlicht, H. & Rabinowitz, J. C. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3278–3282.