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The Binding of Pentaammineruthenium (III) to RNase B and RNase A + d(pA)$_4$ in the Crystalline State

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The binding of pentaammineruthenium (III) to ribonuclease A and B both free and complexed with d(pA)$_4$ has been examined in the crystalline state through the application of X-ray diffraction and difference Fourier techniques. In crystals of native RNase B, the reagent was observed to have many binding sites, some entirely electrostatic in nature and others consistent with coordination to histidine residues. The primary histidine in the latter case was 105 with 119 also partially substituted. In crystals of RNase A + d(pA)$_4$ complex only a single, extremely strong site of substitution was observed, and this was 2.4 Å from the native position of the imidazole ring of histidine 105. Thus, the results of these X-ray diffraction studies appear to be quite consistent with the findings of earlier NMR studies and with the results obtained in crystals of the gene 5 DNA binding protein.

KEY WORDS: RNase B, RNase A + d(pA$_4$); pentaammineruthenium; X-ray crystallography; difference Fourier; histidine modification.

1. INTRODUCTION

The prevalence for ammine complexes of ruthenium in aqueous environments to coordinate with biologically important heterocyclic aromatic ligands such as imidazoles, pyrimidines, purines, pyridines, and isoalloxazines has been well documented (Clarke, 1980*a*; Sundberg and Martin, 1974). In proteins, the predominant mode of binding is to histidine residues, as shown in Fig. 1. Ruthenium complexes have further been shown to demonstrate a range of biological properties, including oncostatic and mutagenic effects, and to induce error-prone DNA repair (Clarke, 1980*b*). Ruthenium pentaammine complexes have been employed as probes in a wide range of physico-chemical studies of both proteins and nucleic acids. X-ray diffraction has been used to examine the complexation of Ru(III)$(NH_3)_5Cl_3$ with yeast phenylalanyl-tRNA (Rubin *et al.*, 1983) and with the gene 5 DNA unwinding protein from bacteriophage fd (Axelrod *et al.*, 1986). Pentaammineruthenium (III) has been used to label histidine 33 in horse heart ferricytochrome c in order to

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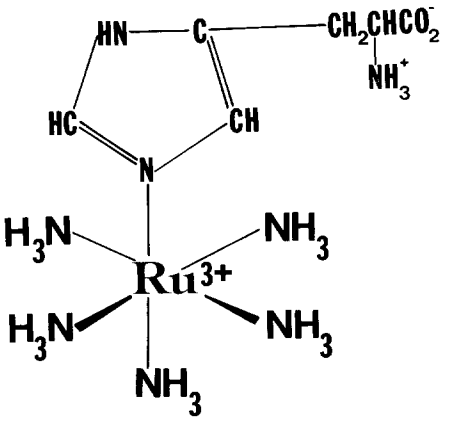

Fig. 1. The mechanism by which proteins bind ruthenium pentaammine reagent is through the imidazole of histidine side chains, which forms a ligand of the coordination complex.

measure the rate of intramolecular electron transfer between the ruthenium and heme redox centers (Winkler *et al.*, 1982; Yocom *et al.*, 1982). Labeling of histidine residues in α-lytic protease and lysozyme permitted measurement of the interresidue distance in proteins through a mechanism involving fluorescence quenching of tryptophan residues by pentaammineruthenium (III) complexes (Recchia *et al.*, 1982).

Pancreatic ribonuclease (RNase A) has been modified by reaction of paramagnetic pentaammineruthenium (III) with its histidine residues, thereby permitting assignment of two histidine proton resonances in the NMR spectrum of the protein (Matthews *et al.*, 1980*a*). It has also been used as an NMR probe to monitor urea-induced conformational changes in RNase A (Matthews *et al.*, 1980*b*).

There are four histidine residues in RNase: histidines 12, 48, 105, and 119. Histidines 12 and 119 are directly at the active site of the enzyme. Although they are labeled slowly in the presence of excess ruthenium, under more stringent conditions, the primary site of substitution is histidine 105. The modification of histidine 105 results in decreased activity of the enzyme toward 2',3' cyclic CMP, a model substrate. The derivatized protein was reported to have 66% the activity of the native protein (Matthews *et al.*, 1980*b*).

In these studies, we examined the reaction specificity of pentaammineruthenium (III) using X-ray diffraction and difference Fourier analysis. The objectives were to characterize further the mode of binding of the complex to protein molecules, to correlate our results with the NMR data, to see whether the complex favored reaction with DNA over protein (or vice versa), and finally to determine whether the compound might be useful for formation of isomorphous heavy-atom derivatives for X-ray crystallography.

We conducted our analyses on two different RNase-containing crystals. The first was a monoclinic crystal of the glycosylated form of ribonuclease, known as RNase B (Plummer and Hirs, 1963; Liang *et al.*, 1980), the structure of which has

recently been solved using X-ray diffraction analysis (manuscript in preparation). The second crystal (McPherson *et al.*, 1986) was of a complex between RNase A and four independent tetramers of $d(pA)_4$. In the first case, the pentaammineruthenium (III) complex was presented with two independent molecules of RNase B in the asymmetric unit of the crystal, allowing us a means of assessing the importance of lattice interactions with regard to ruthenium binding. The second crystal allowed us to determine whether the presence of nucleic acid bound to RNase A altered the pattern of ruthenium reactivity and, at the same time, whether the compound demonstrated a preference, or even an affinity, for the single-stranded DNA. In both cases, we could determine whether the pattern of reactivity, predominantly histidine 105 and then histidines 119 and 12, was the same in the crystal as in solution.

The crystal structure of RNase B has been refined by us to 2.5 Å resolution by constrained-restrained least-squares techniques to a conventional R factor of 0.22. The crystal structure of the RNase A + $d(pA)_4$ complex has been similarly refined to the same resolution and approximately the same R factor (McPherson *et al.*, 1986). The phases calculated from both model structures have been shown in several other of our studies to be quite trustworthy in producing bound ligands of various sorts in difference Fourier syntheses. In addition, it was possible to check substitution sites further by examining difference Patterson maps for the vector distributions predicted by the set of binding sites derived from difference Fourier syntheses.

2. MATERIALS AND METHODS

Crystals of ribonuclease B were grown by combining 10 μl of a 20-mg/ml solution of commercially prepared ribonuclease B (Sigma) in double-distilled water with 10 μl of an 18% (w/w) solution of polyethylene glycol (PEG) 4000 (Sigma). The resulting droplets were allowed to stand in vapor diffusion boxes (McPherson, 1982) containing reservoirs of 18% (w/w) PEG 4000 in double-distilled water. After 4–6 weeks, crystals suitable for diffraction appeared. The crystals grew in space group C2, with $a = 101.81$ Å, $b = 33.36$ Å, $c = 73.6$ Å, and $\beta = 90.4°$.

Crystals of the RNase A + $d(pA)_4$ complex were grown from 16–18% PEG 4000 at 4°C by vapor diffusion as previously described (McPherson *et al.*, 1986) and the structure solved by a combination of multiple isomorphous replacement and molecular replacement techniques. The crystals were of space group $P2_12_12_1$, with $a = 44.64$, $b = 75.3$, and $c = 44.8$ Å.

Pentaammineruthenium (III) trichloride, $Ru(NH_3)_5Cl_3$ was purchased from Johnson Matthey. The crystals were derivatized by slowly adding 2 μl of the saturated $Ru(NH_3)_5Cl_3$ solution to the crystals in samples of approximately 15 μl over a period of 2 days. The crystals were not mounted before another 10 days had passed. The monoclinic RNase B crystals assumed a heavy brown color almost immediately, which did not change appreciably over time. By contrast, the crystals of RNase A + $d(pA)_4$ behaved quite differently. Upon initial addition of the reagent, the crystals remained clear, but this changed to a pale pink color over 1 week, eventually changing to pale violet by the time data collection was begun 2 weeks later. Neither of the two RNase crystals was damaged by the treatment with ruthenium.

X-ray diffraction data to 3.0 Å resolution were collected at 20°C on the ruthenium-derivatized crystals using an Enraf-Nonius CAD-4 automated diffractometer with an extended counter arm. Data were collected using an omega scan of 0.70° width, and the scan rate was 1°/min. The generator, equipped with a standard broad-focus tube, was operated at 40 kV and 32 mA. Friedel pairs were collected throughout, and one crystal of each form was sufficient for a complete data set. Backgrounds were measured on both sides of every scan. An empirical absorption correction curve was obtained by measuring a ϕ-independent reflection at 36 consecutive intervals of 10°. Three standard reflections were collected every 8000 sec of X-ray exposure time to monitor crystal deterioration.

The integrated intensities were corrected for Lorentz polarization effects and absorption, and backgrounds were subtracted. The backgrounds for each reflection were taken as the local average of backgrounds for the 24 nearest neighbours in reciprocal space. The intensites were merged and converted to structure amplitudes. Standard deviations were determined from counting statistics and from merging residuals. The R syms for the merged reflections were very close to 0.05 for reflections greater than 1 standard deviation for all data sets.

The data sets for the ruthenium-derivatized crystals were scaled to the previously recorded native structure amplitudes, using a Fourier–Bessel procedure (Weissman, 1979). Only reflections greater than three times the standard deviation were used in the difference Fouriers, which were computed on a VAX 780 using the fast Fourier transform program of TenEyck *et al.* (1976). For the monoclinic crystals of RNase B, the data set was composed of 3715 reffections and, when scaled to native, it produced a conventional R factor of 0.31, implying either extensive substitution and/or some nonisomorphous change in the contents of the unit cell. The data set for the RNase A + $d(pA)_4$ complex contained 1750 reflections and demonstrated an R factor of 0.14 when scaled to native. The difference Fourier maps were transferred in contoured form to transparencies containing the skeletal structures of the molecules in the unit cells. These were then examined on a light box.

3. RESULTS

A difference Fourier map of the C2 monoclinic crystal of RNase B using coefficients $F_{ruth} - F_{nat}$ and phases calculated from the refined model showed the presence in the crystal of numerous sites of binding. These fell into two classes: sites adjacent to specific histidine residues, and sites proximal to clusters of charged or polar side chains. In Table I, the coordinates of the ten most prominent peaks are given, along with those side chains within 5 Å of the peak.

There are reasonably convincing peaks corresponding to ruthenium atoms (peaks 2, 3, 6, and 10) near histidine 105 of both molecules in the asymmetric unit. In one case, shown in Fig. 2, the distance from peak 10 to the imidazole group is approximately 3 Å and could represent a coordination complex. The same histidine 105 on the second molecule of RNase B has three peaks (2, 3, and 6) associated with it, all at distances of about 5 A. This might indicate multiple orientations or modes of binding of the ruthenium atom to this histidine; if one were to assume small conformational changes in the residue, all might be compatible with coordina-

Table I. The Major Substitution Sites for Ruthenium for Monoclinic Crystals of RNase B[a]

Peak	*x*	*y*	*z*	Nearest side chain
1	0.12	0.69	0.64	Q69, K66, N67, E86
2	0.26	0.44	0.74	Q60′, E69′, H105′
3	0.25	0.16	0.18	H105′, K104′, N103′
4	0.39	0.59	0.94	K31′, oligosaccharide
5	0.37	0.38	0.44	H119
6	0.24	0.34	0.81	H105′, N103′
7	0.30	0.69	0.26	R39, K37, D38, N27
8	0.40	0.63	0.75	N24, Q28, N27, D14
9	0.15	0.44	0.39	K1, E49′
10	0.05	0.41	0.60	H105, N67′, N103

[a] Prime (′) denotes the residue as originating on the second molecule of RNAse B within the asymmetric unit.

tion. We noted, however, that other charged or polar groups were also present nearby, so that the binding could be principally electrostatic.

The only other histidine residue near enough to an apparent ruthenium site was histidine 119 of one protein molecule, but this was not true of the same residue on the second molecule of RNase B. This site was also sufficiently close to be coordinated, about 4 Å, from the imidazole of histidine 119, and no other polar groups were nearby.

All the other major ruthenium sites in the difference Fourier were associated with groups of charged and polar side chains, as evidenced in Table I. Chiefly, these were carboxyl groups of glutamic and aspartic acids, carboxyamides of asparagine and glutamine, and occasionally the positively charged groups of lysine and arginine. It was frequently, observed that the ruthenium atom was disposed at the centers of various groups of these residues in a manner suggesting ionic interactions. The most outstanding among these (peak 1 in Table I), shown in Fig. 3, lies

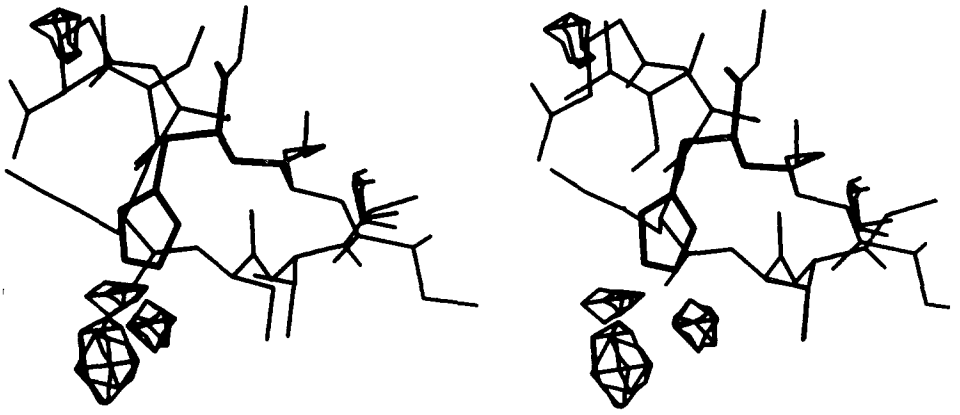

Fig. 2. Computer-generated stereo image of the difference electron density due to bound ruthenium complex superimposed on the protein structure of RNase B in the immediate vicinity of histidine 105 of one of the two molecules within the crystallographic unit cell.

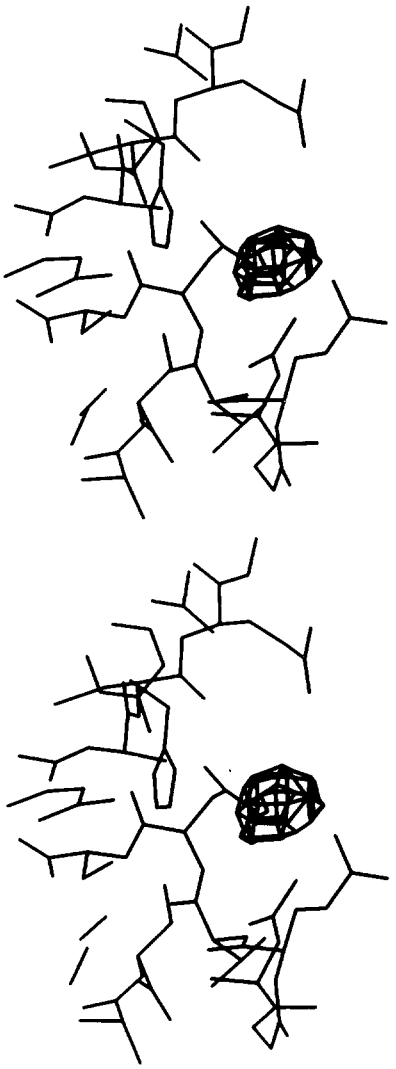

Fig. 3. Stereo image of the highest electron-density peak in the difference Fourier map (peak 1 of Table I) lying at the center of a cluster of hydrophilic residues. The side chains forming the constellation are contributed by two different RNase B molecules within the crystallographic unit cell.

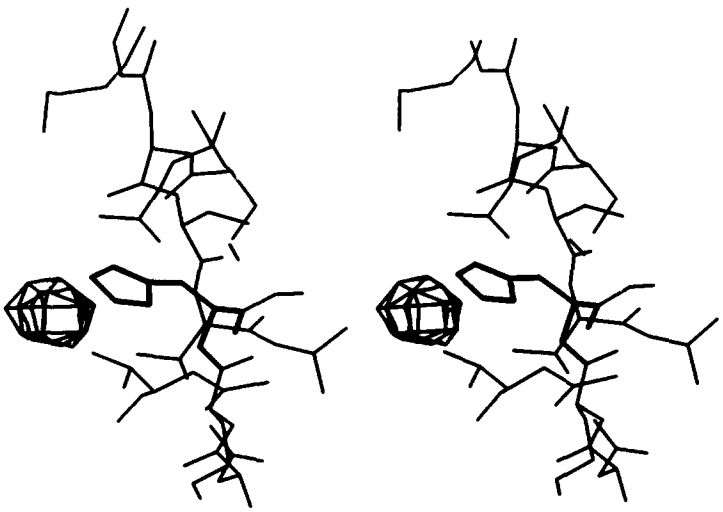

Fig. 4. The single peak, 2.5 times the height of any other point in the difference Fourier map, superimposed on the RNase A+d(pA)$_4$ complex structure in its immediate neighborhood. The distance to the imidazole group of histidine 105 is 2.4 Å, entirely consistent with coordination complex.

in a solvent region at the center of a cluster composed of Q69, K66, N67, and E86′. The ruthenium is clearly bound through electrostatic interactions and serves to form a bridge linking two independent molecules of RNase B in the lattice.

Results of the difference Fourier map of the $P2_12_12_1$ crystal of RNase A+d(pA)$_4$ sere both straightforward to interpret and unequivocal. It contained only a single intense peak 2.5 times the height of any other point on the map and about 12 times the estimated standard deviation for the difference Fourier. As shown in Fig. 4, it was located 2.4 Å from the native position of the imidazole ring of histidine 105. There was no negative density to suggest any movement of this side chain, nor was there density anywhere in the map to suggest a significant change in structure of either the protein or the DNA.

4. DISCUSSION

The crystal of RNase A+d(pA)$_4$ treated with pentaammineruthenium was clearly very isomorphous and produced a near-perfect difference Fourier map. The major and apparently only site of substitution was on the imidazole of histidine 105. We observed no reactivity elsewhere, including histidines 12 and 119 or at any location on the DNA. The complete lack of reactivity of histidines 12 and 119 was likely due, in this case, to protection by DNA which prevented access by the ruthenium complex. In agreement with the NMR studies conducted by Matthews *et al.* (1980*a*), we find histidine 105 to be a strongly reactive site and to be the most probable major substitution site in solution as well. We also note that the crystal coordination with histidine 105, shown in Fig. 5, was accomplished with no

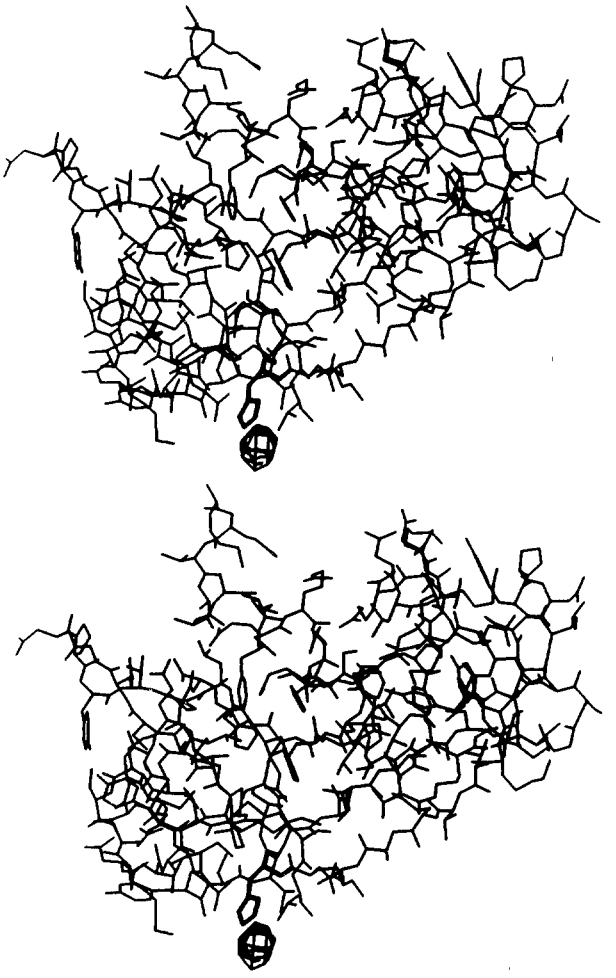

Fig. 5. Stereo image of the ruthenium peak shown in Fig. 4 superimposed on the entire molecule of RNase A, with histidine 105 emphasized. The $d(pA)_4$ component of the complex is not shown.

conformational change in the protein molecule. Ruthenium pentaammine did not react with any of the 16 adenosine groups presented by the four $d(pA)_4$ oligomers. Thus, it appears that protein is strongly preferred over this DNA component.

The situation with the monoclinic crystal of RNase B was, as one might have predicted, more complicated. Although there was no evidence of extensive non-isomorphism, there were many more sites of binding. This increase was due in part to the fact that there were two molecules of RNase B per asymmetric unit of the crystal. As with the RNase A+$d(pA)_4$ crystal, a major site of substitution was histidine 105 on both molecules within the asymmetric unit. Whereas binding of ruthenium to one of these molecules was accomplished without any perceptible change in side-group disposition, on the second molecule the histidine residue may have moved from 2 to 3 Å in order to accommodate the ruthenium pentaammine ligand and may have assumed multiple orientations, depending on the direction of approach of the ruthenium complex.

The extent of ionic complex formation in contrast to coordination with histidine residues was clearly greater for the unliganded RNase B molecules than for RNase A complexed with nucleic acid, where there was no apparent binding except by the coordination mode. We believe this may be explained to some degree by differences in packing in the two crystals, by differences in reaction conditions in the two crystals (as evidenced by the very different colors produced in the crystals by the ruthenium reagent), and also by the relatively substantial protection afforded the RNase A by the bound fragments of DNA. It seems clear from these experiments that ruthenium pentaammine can react with proteins in both a highly specific manner or in a rather nonspecific fashion, depending on the conditions and state of the macromolecule. It also appears, however, that even when ionic binding does predominate, some coordination to histidines takes place. In addition, the results with RNase A+$d(pA)_4$ in the crystal exactly parallel those of Matthews in solution in that the reaction under limiting conditions is quite specific in RNase A for histidine 105 (Matthews *et al.*, 1980*a*,*b*). The results with RNase B are also consistent with Matthews' result in that primary sites of reaction are again histidine 105, with subsidiary reaction at histidine 119.

Because of the preference for histidine residues shown by pentaammineruthenium for RNase and elsewhere for the gene 5 DNA-binding protein (Axelrod *et al.*, 1986), we believe that the compound may be useful as a heavy-atom derivative for protein crystallography. This will be particularly true for proteins lacking other reactive groups, such as cysteines, and those that have a small number of histidine residues. We were also pleased to find that the pattern and order of histidine reactivity was entirely consistent with that determined in solution using NMR. Thus, this stands as further evidence, if any were needed, that the crystallization process does little to affect the properties of a protein with respect to those in solution.

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REFERENCES

Axelrod, H., DeLozier, G., Greene, S., and McPherson, A. (1986). *J. Protein Chem.* **4**, 235–243.

Clarke, M. J. (1980*a*). In *Metal Ions in Biological Systems* (Siegel, H., ed.), Vol. 11, Marcel Dekker, New York, pp. 231–283.

Clarke, M. J. (1980*b*). In *Inorganic Chemistry in Biology and Medicine*, American Chemical Society Symposium Series, Vol. 140, pp. 157–180

Liang, C., Yamashita, K., and Kobata, A. (1980). *J. Biochem.* (*Tokyo*) **88**, 51–58.

Matthews, C. R., Recchia, J., and Foebe, C. (1980*a*). *Anal. Biochem.* **112**, 329–337.

Matthews, C. R., Erickson, P. M., and Forebe, C. L. (1980*b*). *Biochim. Biophys. Acta* **624**, 4499–4510.

McPherson, A. (1982). *The Preparation and Analyses of Protein Crystals*, Wiley, New York.

McPherson, A., Brayer, G. D., and Morrison, R. (198). *J. Mol. Biol.* **189**, 305–327.

Plummer, T. H., and Hirs, C. (1963). *J. Biol. Chem.* **238**, 1396–1401.

Recchia, J., Matthews, C. R., Rhee, M.-J., and Horrucks, W. D., Jr. (1982). *Biochim. Biophys. Acta* **702**, 105–111.

Rubin, J. R., Sabat, M., and Sundaralingam, M. (1983). *Nucleic Acids Res.* **11**, 6571–6586.

Sundberg, R. J., and Martin, B. R. (1974). *Chem. Rev.* **74**, 471–517.

TenEyck, L., Weaver, L. H., and Matthews, B. W. (1976). *Acta Crystallogr.* **A32**, 349.

Weissman, L. (1979). Dissertation, Progress in the determination of the structure of crystalline rabbit muscle aldolase, University of California, Los Angeles.

Winkler, J. R., Nocera, D. G., Yocom, K. M., Brodignon, E., and Gray, H. B. (1982). *J. Am. Chem. Soc.* **104**, 5800–5801.

Yocom, K., Shelton, J. B., Shelton, J. R., Schroeder, W. A., Worosila, G., Isied, S. S., Bordignon, E., and Gray, H. B. (1982). *Proc. Natl. Acad. Sci. USA* **79**, 7052–7055.