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Transient Spectroscopy of the Reaction of Cyanide with Ferrous Myoglobin

EFFECT OF DISTAL SIDE RESIDUES*

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The reaction of cyanide metmyoglobin with dithionite conforms to a two-step sequential mechanism with formation of an unstable intermediate, identified as cyanide bound ferrous myoglobin. This reaction was investigated by stopped-flow time resolved spectroscopy using different myoglobins, *i.e.* those from horse heart, *Aplysia limacina* buccal muscle, and three recombinant derivatives of sperm whale skeletal muscle myoglobin (Mb) (the wild type and two mutants). The myoglobins from horse and sperm whale (wild type) have in the distal position (E7) a histidyl residue, which is missing in *A. limacina* Mb as well as the two sperm whale mutants (E7 His → Gly and E7 His → Val).

All these proteins in the reduced form display an extremely low affinity for cyanide at pH < 10. The differences in spectroscopy and kinetics of the ferrous cyanide complex of these myoglobins indicate a role of the distal pocket on the properties of the complex. The two mutants of sperm whale Mb are characterized by a rate constant for the decay of the unstable intermediate much faster than that of the wild type, at all pH values explored. Therefore, we envisage a specific role of the distal His (E7) in controlling the rate of cyanide dissociation and also find that this effect depends on the protonation of a single ionizable group, with pK = 7.2, attributed to the E7 imidazole ring.

The results on *A. limacina* Mb, which displays the slowest rate of cyanide dissociation, suggests that a considerable stabilizing effect can be exerted by Arg *E*10 which, according to Bolognesi *et al.* (Bolognesi, M., Coda, A., Frigerio, F., Gatti, C., Ascenzi, P., and Brunori, M. (1990) *J. Mol. Biol.* 213, 621–625), interacts inside the pocket with fluoride bound to the ferric heme iron.

A mechanism of control for the rate of dissociation of cyanide from ferrous myoglobin, involving protonation of the bound anion, is discussed.

In recent years the concerted efforts of crystallography, molecular dynamics, transient spectroscopy, and genetic engineering have contributed to a quantitative description and a deeper understanding of ligand binding in hemoglobin and myoglobin, including the structural control exerted by the protein on the activation barriers. Recent experiments on myoglobin and hemoglobin mutants obtained by means of site-directed mutagenesis of these proteins (1, 2) have shown that the dynamics of oxygen dissociation are controlled by interactions with the distal His-E7 (3). These results support previous information obtained on sperm whale Mb[1] by EPR (4), x-ray crystallography (5), and neutron diffraction (6) indicating that oxygen is stabilized by H-bonding to the His-E7.

In this paper we report on the kinetics of buildup and dissociation of the complex of cyanide with different ferrous myoglobins, *i.e.* horse heart Mb and sperm whale (SW) skeletal muscle Mb (both containing His-E7); the mutants of SW Mb with Gly-E7 and Val-E7 (obtained by site-directed mutagenesis and expression in *Escherichia* coli, see Ref. 2); and *Aplysia limacina* Mb (which has Val-E7 at the distal site position, see Refs. 7 and 8). It is known that the affinity of cyanide for ferrous Hb and Mb is very low ($K_d > 0.1$ M even at very high pH; see Refs. 9 and 10); however, rapid reduction (by $Na_2S_2O_4$) of the cyanide complex of ferric Hb and Mb (Hb^+CN^- or Mb^+CN^-) leads to the formation of an unstable intermediate which slowly dissociates yielding free HCN and reduced Hb or Mb, as indicated by transient kinetic studies reported by others (11, 12).

The kinetics of decay of the intermediate from myoglobin mutants may provide new valuable information on the mechanism(s) which controls ligand dissociation rates. Using stopped-flow time-resolved spectroscopy, we show that the optical spectrum of the cyanide complex of ferrous Mb and the rate of dissociation of cyanide vary considerably with the nature of the distal pocket residues. For the two Mbs (horse and SW) with His-E7, the cyanide dissociation rate constant is considerably lower than that of the two mutants of sperm whale at all pH values from 9.2 to 5. In the case of *A. limacina* Mb (Val-E7), the same constant is essentially pH independent and even slower than in the other Mbs.

A mechanism whereby dissociation is assisted by protonation of bound CN^-, based on the pH dependence of the reaction in horse and SW Mbs, is discussed.

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[1] The abbreviations used are: Mb, myoglobin; SW, sperm whale.

MATERIALS AND METHODS

Myoglobin from horse heart was purchased from Sigma and repurified by ammonium sulfate fractionation (with cuts at 70 and 95% saturation, see Ref. 13). Sperm whale Mb wild type and two distal histidine mutants (E7 His → Gly, and E7 his → Val), all having N-terminal Met, were expressed in *E. coli*, as reported by Sligar and coworkers (2, 14).

Myoglobin from *A. limacina* buccal muscle was prepared as described by Rossi-Fanelli and Antonini (15).

Protein concentrations were determined spectrophotometrically employing a Cary 219 spectrophotometer and using published extinction coefficients for the cyanide derivative of ferric myoglobin (13).

All reagents were of analytical grade.

A typical stopped-flow experiment was carried out as follows. A 2–20 μM solution of ferric myoglobin (Mb^+) in the presence of 1 mM KCN was mixed in the Gibson-Durrum stopped-flow apparatus with 1–100 mM sodium dithionite ($Na_2S_2O_4$). The absorption spectrum was recorded (over a 150-nm wavelength range) by means of a rapid scanning photodiode array spectrophotometer (Tracor Northern TN6500) adapted to the 2-cm light path cell of the stopped-flow apparatus. The Tracor rapid scanning spectrophotometer acquires 512/1024 photodiode counts in 5/10 ms and stores up to 64 spectra. The time resolution and spectroscopic performance of TN6500 were carefully checked by comparison with absolute and difference spectra obtained both with the Cary 219 spectrophotometer and with the conventional Gibson-Durrum single wavelength stopped-flow spectrophotometer equipped with an Olis aquisition system (stopped-flow operating system, OLIS, Jefferson, GA).

TN6500 data sets were transferred to a CompaQ Deskpro 286 or to a MicroVAX 3500 for further analysis. Time courses of the optical changes recorded at different wavelengths or different pH values (5–9.2) were simultaneously fitted to a series of exponentials, using the equation developed by Bateman (16) and treating the reaction as irreversible 1st order processes.

RESULTS

Fig. 1 depicts the time-resolved difference spectra calculated from the photodiode counts recorded from flow stop to 20 s after mixing $Na_2S_2O_4$ with Mb^+CN^- at pH 7 and 20 °C. The two data sets refer to wild-type SW Mb His-E7 and mutant SW Mb Gly-E7. The wavelength range is 500–650 nm; the initial spectrum corresponds to that of Mb^+CN^- and the final one to that of ferrous Mb. Similar data sets over the visible or the Soret regions were obtained for all the proteins at different pH values.

The overall time evolution can be described, in all cases, by the following two-step reaction scheme:

$$Mb^+CN^- \xrightarrow{k_1} \text{intermediate} \xrightarrow{k_2} Mb + HCN \qquad \text{Scheme 1}$$

The first reaction is the reduction of the ferric iron driven by $Na_2S_2O_4$ in excess; this process is $Na_2S_2O_4$ concentration dependent (although of order lower than 2, see below). The second reaction conforms to a simple irreversible monomolecular decay of the intermediate and is often much slower than the reduction of the heme iron. Simultaneous fit of the experimental data at different wavelengths to such a model is depicted in Fig. 2. Analysis shows that the intermediate is a unique and well identified species and the fit of the data allows calculation of its spectrum. The visible spectrum for the intermediate in the case of SW Mb His-E7, SW Mb Gly-E7, and *A. limacina* Mb (see Fig. 3) is characterized by two main bands, recalling those reported for human Hb and SW Mb (9–11).

When the same experiments were carried out in the presence of CO (0.5 mM after mixing), thereby leading to MbCO as the final product, the observed time course was quantitatively described by the same rate constants.

The first step (Scheme 1) has the same rate constant for SW Mb His-E7 and SW Mb Gly-E7, while the second is considerably slower when His-E7 is present; the same applies

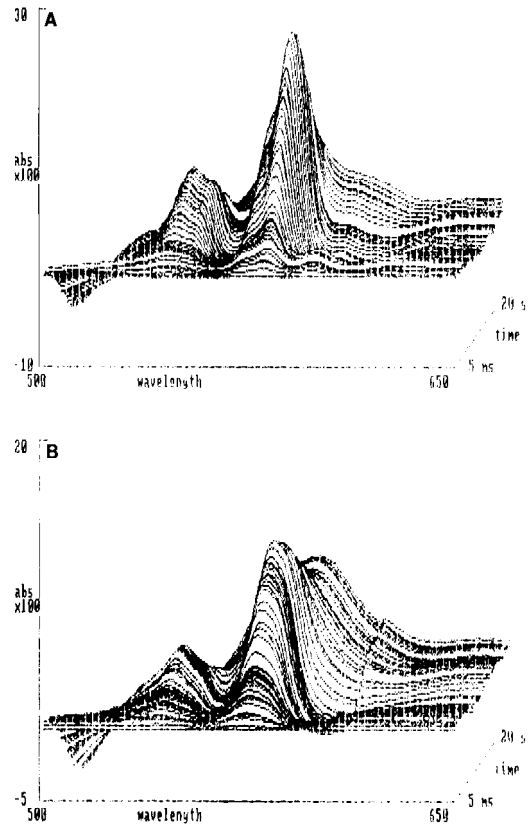

FIG. 1. **Time-resolved difference spectra recorded during the reduction of cyano-met Mb.** *A*, sperm whale Mb His-E7. *B*, sperm whale Mb Gly-E7. Experimental conditions: cyano-met Mb (18 μM) in 0.1 M phosphate buffer, pH 7, plus 1 mM cyanide was mixed with 100 mM dithionite; temperature was 20 °C. The spectra were recorded at different times after mixing using a non-linear acquisition scheme. The first spectrum recorded (corresponding to that of $Mb(Fe^{+3})CN^-$) was used as baseline.

to the SW Mb Val-E7 mutant. In horse Mb (His-E7) reduction is somewhat faster, but cyanide dissociation is comparable to SW Mb His-E7. In *A. limacina* Mb, the buildup of the intermediate is faster and its decay considerably slower than for all other proteins.

The reaction has been studied between pH 5 and 9.2. The results show that both processes (Scheme 1) tend to be faster at acid pH; the pH dependence of the rate constant (k_2) for the decay of the intermediate is shown in Fig. 4. Analysis of the time course of the reaction according to Scheme 1 only yields unequivocal values for the two rate constants when the two phases are well resolved. While this is often the case, SW Mb Gly-E7 dissociates the ligand with a rate which, at acid pH values, is comparable to the rate of reduction. Since in the case of *A. limacina*, horse, and SW Mb His-E7 the spectrum of the intermediate is strictly pH independent, it was assumed to be pH independent also in the case of SW Mb Gly-E7. Thus, all the experiments carried out with this mutant were fitted simultaneously allowing the rate constants to vary with pH but constraining the optical spectrum of the three species to be pH independent. This procedure described satisfactorily and unequivocally all the data.

DISCUSSION

The overall reaction of Mb^+CN^- with dithionite conforms under all conditions to a two-step sequential reaction scheme, in good agreement with previous results (11, 12). The first reaction is a $Na_2S_2O_4$ concentration-dependent process; in agreement with Lambeth and Palmer (17), SO_2^- (a dissociation

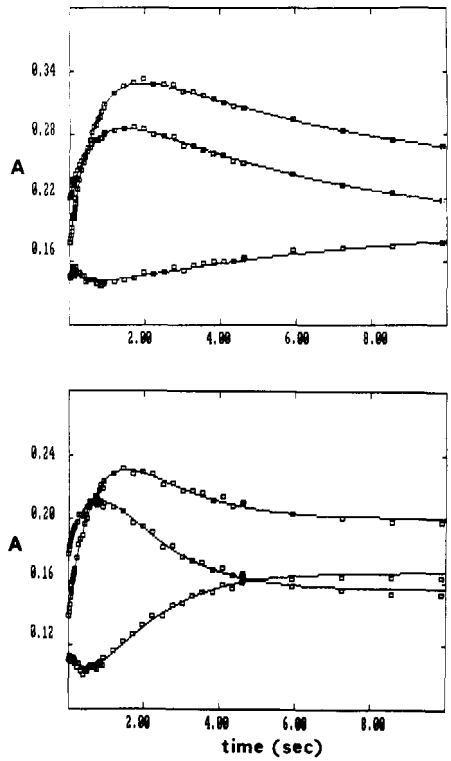

FIG. 2. **Fit of the time course at 480, 568, and 535 nm for the reactions in Fig. 1.** Experimental conditions as in Fig. 1. The two-step sequential mechanism used to fit the data is described in the text.

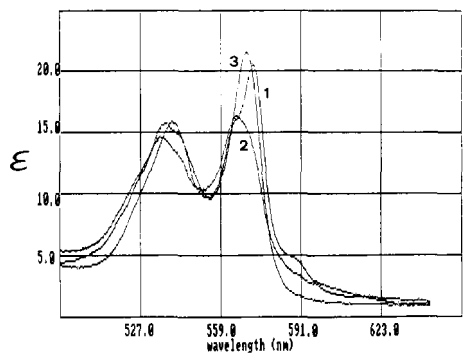

FIG. 3. **Absorption spectra of the intermediates for sperm whale Mb His-E7 (*1*), sperm whale Mb Gly-E7 (*2*), and *A. limacina* Mb (*3*).** The relative populations of $Mb(Fe^{+3})CN^-$ and $Mb(Fe^{+2})$ at time = 1 s (highest population of the intermediate) were calculated and their contributions subtracted. Experimental conditions as in Fig. 1.

product of dithionite) is the reducing species, as demonstrated by the linear dependence of k_1 on the square root of $Na_2S_2O_4$ concentration (not shown). In all cases, reduction becomes faster at lower pH values, suggesting that electrostatic interactions govern the binding of SO_2^- to a site on the protein. However, it is clear that the local charge at the putative binding site is the controlling factor because there is no correlation between the value of k_1 and the isoelectric point of the protein. Thus, horse Mb (isoelectric point = 6.8, see Ref. 18), displays a rate constant for reduction lower than that of *A. limacina* Mb, which has the lowest isoelectric point = 4.5 (18).

The intermediate corresponds to a complex of ferrous Mb with cyanide on the basis of three experimental evidences: (i) the $Na_2S_2O_4$ concentration dependence of the rate constant for the buildup of the intermediate; (ii) the formation of the

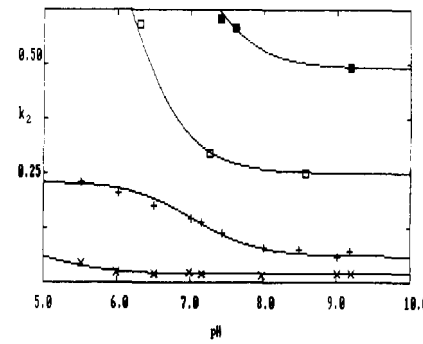

FIG. 4. **pH dependence of k_2, the dissociation rate constant of the ferrous Mb-cyanide complex.** ■, sperm whale Gly-E7 Mb; □, sperm whale Val-E7 Mb; +, horse Mb; ×, *A. limacina* Mb. In the case of horse Mb, the pH dependence was fitted to a single group titration curve with $pK = 7.2$; in the cases of *A. limacina* and sperm whale mutants no fit could be obtained in view of the uncertainty of the upper asymptote, if present at all.

same spectral species upon addition of KCN to ferrous Mb at high pH (in agreement with Ref. 10); and (iii) the fact that carbon monoxide binding is rate limited by dissociation of cyanide from the intermediate, whose spectrum is the same independently of the presence of CO.

In so far as the chemistry of the ligand bound to the intermediate, it cannot be excluded that protonated hydrogen isocyanide may be the species bound to the heme iron, as briefly discussed by Keilin and Hartree (10). However, we are inclined to reject this possibility, as already suggested by Reiseberg and Olson (19), and assume that the actual ligand is the cyanide ion because, among other observations, this best accounts for the pH dependence of ligand release observed in horse and sperm whale myoglobins, which are essentially deprived of Bohr effect for oxygen (13).

In discussing the decay of the ferrous cyanide derivative of Mb, one should recall that all hemoproteins in the ferric form bind cyanide with a very high affinity and a small dissociation rate constant (13). An intermediate will be populated when cyanide dissociation from both the ferric and ferrous forms of the hemeprotein is slower than reduction of the heme iron by dithionite. The above conditions apply to the experiments reported in the present study. It may be noticed, however, that other single chain hemeproteins display different behavior as shown by the cases of leghemoglobin (20) and *Glycera dibranchiata* Hb. In the latter case, upon mixing the met cyanide complex with dithionite, the unliganded ferrous derivative is obtained within 10 ms, without accumulation of an intermediate.[2]

The data reported above show very clearly that the optical spectrum and the kinetic properties of the intermediate are distinctly different for various myoglobins. The spectroscopic and chemical properties of this derivative were described for human hemoglobin (9), sperm whale myoglobin (10, 11), peroxidase (10) and, to a lesser extent, leghemoglobin (20). There have been no attempts, however, to correlate the properties of this derivative with the structure of the heme environment. The comparative analysis of different myoglobins reported here shows that the protein matrix exerts an important control on the spectrum and the rate of cyanide dissociation, making this ligand a sensitive probe for distal interactions.

Horse and SW Mb His-E7 have very similar spectra and kinetics. Comparison of SW Mb His-E7 with the mutant SW Mb Gly-E7 (Fig. 3) shows that the α-band particularly is

[2] A. Bellelli, G. Antonini and M. Brunori, unpublished results.

modified by this single amino acid substitution, the transition at 579 nm clearly seen in the wild type being much less pronounced in the mutant. The spectrum of *A. limacina* Mb is again characteristic.

Comparison of the rate constants for the dissociation of cyanide from the intermediate yields interesting information. The fastest rates are recorded for the mutant SW Mb Gly-E7, which for example at pH = 9 displays a value five times faster than that of SW Mb His-E7 (k_2 = 0.09/s). The other mutant, with Val on the distal site, displays an intermediate behavior, with a rate constant at pH = 9 three times faster than that of the wild type. In these mutants the amino acid side chains on the distal side cannot establish suitable interactions with the bound ligand, as indicated also by the very rapid kinetics of oxygen dissociation, which for the SW Mb Gly-E7 is 100 times faster than for wild type (3, 21). Therefore, we conclude that His-E7 interacts with the bound cyanide and thereby stabilizes the complex.

The data in Fig. 4 show that dissociation is faster at acid pH values and suggest as a possible mechanism for dissociation protonation of the anion in the pocket. Horse Mb displays a clean pH dependence of k_2 (Fig. 4), with a pK = 7.2; the pH dependence of SW Mb His-E7 is similar to that of horse although no pK has been determined in view of the limited data available. The pH titration is fully consistent with a mechanism involving protonation of bound cyanide mediated by protonation of the distal His, which acts as a relay. This is the same mechanism which was proposed to account for the rapid kinetics of protonation of OH^- bound to the ferric iron in SW Mb (22). The two mutants, besides being faster, display a pH dependence of k_2 which is different from that of the wild type in so far as the increase in the rate for cyanide dissociation brought about by lowering pH occurs below 7.2 (see Fig. 4). The large increase in the value of k_2 seen in both mutants at acidic pH values excludes a unique role of His-E7 in controlling the protonation of bound cyanide and supports the hypothesis that this process may occur in the two mutants directly from the bulk, possibly related to a bigger space in the cavity.

The case of myoglobin from *A. limacina*, which displays the slowest (largely pH independent) rate constant for dissociation of bound cyanide, demands a separate discussion. This protein also lacks a distal His, and the three-dimensional structure (8) shows that Val-E7 occupies the distal site. Recent crystallographic results by Bolognesi *et al.* (23) have shown that in *A. limacina* $Mb^+ F^-$, Arg-E10 swings into the distal pocket and becomes part of a network of H-bonds stabilizing the bound ligand. This novel interaction is assumed by Bolognesi *et al.* (23) to be present also in the oxygenated derivative of the same protein, thus accounting for the fact that its oxygen dissociation rate constant (24) is six times faster than horse or SW Mb His-E7, but still 20 times slower than the mutant SW Mb Gly-E7 (3). Since Arg is expected to interact with a bound anion but not to favor proton transfer, it may produce a considerable stabilizing effect. This hypothesis may be subject to verification by crystallographic analysis of the cyano met derivative of *A. limacina* met Mb. It may be noticed that protonation of bound OH^- in ferric *A. limacina* Mb is exceedingly slow (25) compared with the same process for SW Mb (22) because of the lack of proton transfer from the distal His which represents, in our view, another piece of evidence to support the model discussed above.

In conclusion, we have characterized by spectroscopy and kinetics the complex of ferrous Mb with cyanide and the role of the distal pocket, where amino acid side chains may (or may not) interact with the bound cyanide changing the spectrum of the liganded species and controlling the rate of dissociation. At every pH, the fastest rate of dissociation was recorded for the mutant of SW Mb with Gly-E7. A possible mechanism of dissociation involves protonation of the bound CN^-, which may occur directly from the bulk or via the distal His-E7, where present in the protonated state. In the case of *A. limacina* Mb, which lacks a distal His, stabilization of bound cyanide may be due to Arg-E10, as suggested by Bolognesi *et al.* (23) for the fluoride ion bound to the ferric derivative.

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REFERENCES

1. Nagai, K., and Perutz, M. F. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 7252–7255
2. Springer, B. A., and Sligar, S. G. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 8961–8965
3. Olson, J. S., Mathews, A. J., Rohlfs, R. J., Springer, B. A., Egeberg, K. D., Sligar, S. G., Tame, J., Renaud, J. P., and Nagai, K. (1988) *Nature* **336,** 265–266
4. Ikeda-Saito, M., Iizuka, T., Yamamoto, H., Kayne, F. J., and Yonetani, T. (1977) *J. Biol. Chem.* **252,** 4882–4887
5. Phillips, S. E. V. (1980) *J. Mol. Biol.* **142,** 531–554
6. Phillips, S. E. V., and Schoenborn, B. P. (1981) *Nature* **292,** 81–82
7. Tentori, L., Vivaldi, G., Carta, S., Marinucci, M., Massa, A., Antonini, E., and Brunori, M. (1973) *Int. J. Pept. Protein Res.* **5,** 187–200
8. Bolognesi, M., Onesti, S., Gatti, G., Coda, A., Ascenzi, P., and Brunori, M. (1989) *J. Mol. Biol.* **205,** 529–544
9. Stitt, F., and Coryell, C. D. (1939) *J. Am. Chem. Soc.* **61,** 1263–1266
10. Keilin, D., and Hartree, E. F. (1955) *Biochem. J.* **61,** 153–171
11. Cox, R. P., and Hollaway, M. R. (1977) *Eur. J. Biochem.* **74,** 575–587
12. Olivas, E., De Waal, D. J. A., and Wilkins, R. G. (1977) *J. Biol. Chem.* **252,** 4038–4042
13. Antonini, E., and Brunori, M. (1971) *Hemoglobin and Myoglobin in their Reactions with Ligands*, North-Holland, Amsterdam
14. Springer, B. A., Egeberg, K. D., Sligar, S. G., Rohlfs, R. J., Mathews, A. J., and Olson, J. S. (1989) *J. Biol. Chem.* **264,** 3057–3060
15. Rossi-Fanelli, A., and Antonini, E. (1957) *Biochimia (Moscow)* **22,** 336–345
16. Bateman, H. (1910) *Proc. Cambridge Phyl. Soc.* **15,** 423–427
17. Lambeth, D. O., and Palmer, G. (1973) *J. Biol. Chem.* **248,** 6095–6103
18. Rossi-Fanelli, A., Antonini, E., and Povoledo, D. (1957) in *Symposium on Protein Structure* (Neuberger, A., ed) pp. 144–147, Methuen & Co. Ltd., London
19. Reiseberg, P. I., and Olson, J. S. (1980) *J. Biol. Chem.* **255,** 4144–4150
20. Appleby, C. A., Wittenberg, B. A., and Wittenberg, J. A. (1973) *J. Biol. Chem.* **248,** 3183–3187
21. Rohlfs, R. J., Mathews, A. J., Carver, T. E., Olson, J. S., Springer, B. A., Egeberg, K. D., and Sligar, S. G. (1990) *J. Biol. Chem.* **265,** 3168–3176
22. Ilgenfritz, G., and Schuster, T. M. (1971) *Probes of Structure and Function of Macromolecules and Membranes*, Vol. 2, pp. 399–406, Academic Press, New York
23. Bolognesi, M., Coda, A., Frigerio, F., Gatti, C., Ascenzi, P., and Brunori, M. (1990) *J. Mol. Biol.* **213,** 621–625
24. Wittenberg, B. A., Brunori, M., Antonini, E., Wittenberg, J. B., and Wyman, J. (1965) *Arch. Biochem. Biophys.* **111,** 576–579
25. Giacometti, G. M., Da Ros, A., Antonini, E., and Brunori, M. (1975) *Biochemistry* **14,** 1584–1588