Fourier Transform Infrared Evidence against Asp β99 Protonation in Hemoglobin: Nature of the Tyr α42−Asp β99 Quaternary H-Bond[†]

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ABSTRACT: The Tyr α42−Asp β99 intersubunit H-bond stabilizes the T quaternary structure in hemoglobin (Hb) tetramers. We had proposed that Tyr α42 acts as an acceptor in this H-bond, because the tyrosine Y8a/8b and Y7a′ UVRR (ultraviolet resonance Raman) bands shift in directions opposite to those expected if tyrosine is an H-bond donor. If Asp β99 is the H-bond donor, then it must be protonated in the T state, and would be a previously unrecognized contributor to the Bohr effect. This implication was strengthened by the discovery that an R-minus-T difference FTIR (Fourier transform infrared) band at 1693 cm^{-1}, which might be a signal from protonated carboxylate, is missing in Hb Kempsey, a mutant in which Asp β99 is replaced by Asn. However, we now find that this FTIR signal is insensitive to ^{13}C-labeling of the aspartate residues in Hb, and cannot arise from protonated Asp β99. There are no other difference signals in the 1700 cm^{-1} region at a sensitivity of one COOH group. We conclude that Asp β99 is *not* protonated, and that the anomalous UVRR shifts must arise from compensating polarization of the Tyr α42 OH. Candidates for this compensation are the H-bond donated by the Asp β94 backbone NH, and the nearby positive charge of Arg β40.

Perutz and TenEyck (*1*) long ago noted a key structural element in the deoxyHb[1] tetramer, namely, an intersubunit H-bond between the side chains of Tyr α42 and Asp β99. The interaction was proposed to be a key stabilizing element in the T quaternary structure, accounting for a part of the free energy of cooperativity. Consistent with this postulate is the substantial increase in oxygen affinity, and decrease in cooperativity, observed for Hb mutants in which either Tyr α42 or Asp β99 is replaced [(*2*), (*3*) and references cited therein]. The best-studied of these mutants is Hb Kempsey, in which Asp β99 is replaced by asparagine. It exhibits high oxygen affinity ($p_{50} = 0.23$ Torr compared to 2.4 Torr for Hb A) and essentially no cooperativity (Hill coefficient $n = 1.1$ compared to $n = 2.6$ for Hb A) at physiological pH (*4*). These changes are partially recovered by addition of allosteric effectors, such as inositol hexaphosphate (IHP), which stabilize the T state. Thus replacement of the Asp β99 carboxylate side chain with a carboxamide group destabilizes the T state by weakening the interaction with Tyr α42.

Following Perutz and TenEyck (*1*), this interaction has always been assumed to involve H-bond donation from the Tyr α42 OH to the Asp β99 carboxylate anion. However, this assumption was called into question by the discovery (*5*) that the 230 nm-excited ultraviolet resonance Raman (UVRR) Tyr Y8a/8b signals failed to show the downshift that is known to accompany H-bond donation by the tyrosine OH group (*5*, *6*). Instead the bands shifted up in the T state, a direction implying H-bond acceptance. This inference was supported by a second UVRR study (*7*), using 210 nm excitation, where enhancement is observed for the Y7a′ band, which directly involves the phenyl-OH stretching coordinate. Its frequency is known to increase when the tyrosine OH is an H-bond donor and to decrease when it is an acceptor (*8*). This band was found to shift down in the T state of Hb A.

If Tyr α42 is an H-bond acceptor, what is the donor? Asp β99 seemed the answer (*5*), because in the T state its side chain is inaccessible to solvent, and might well be protonated, even at elevated pH. Positive evidence for Asp β99 protonation was found in the R-minus-T difference FTIR (Fourier transform infrared) spectrum of Hb A (*9*), which showed a 1693 cm^{-1} band in D_2O buffer. This band was missing in Hb Kempsey, even in the presence of IHP. The 1693 cm^{-1} frequency is in the C═O stretching region. It is high for an amide group, and might possibly arise from a carboxylic acid. The (C═O)OH stretch is generally above 1700 cm^{-1} and has been detected in protein IR spectra [see, e.g., (*10*, *11*)], but the frequency is dependent on the H-bond status. Strong H-bonding from an adjacent water molecule might depress the frequency to 1693 cm^{-1}.

Seeking confirmation of this assignment, we have labeled the aspartate side chains, using bacterially expressed Hb. If the 1693 cm^{-1} band does arise from the Asp β99 (C═O)-OH stretch, then it should shift down strongly (~40 cm^{-1}) upon ^{13}C substitution in the carboxylate group (*10*). We now report that this band is unshifted, thereby disproving the proposed assignment. A search for other (C═O)OH FTIR difference bands was negative. We therefore conclude that Asp β99 is *not* protonated, and that its carboxylate anion

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[1] Abbreviations: Hb, hemoglobin; Hb A, adult human hemoglobin; rHb, recombinant human hemoglobin; NA rHb, natural-abundance recombinant hemoglobin; [4-^{13}C]-Asp-rHb, [4-^{13}C]-Asp-labeled recombinant hemoglobin; IHP, inositol hexaphosphate; FTIR, Fourier transform infrared; UVRR, ultraviolet Resonance Raman.

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does indeed accept an H-bond from the Tyr α42 OH, as envisioned by Perutz and TenEyck (*1*). Some other explanation is required for the anomalous Tyr Y8a/8b and Y7a′ shifts in the UVRR spectra. These clearly indicate a positive polarization of the tyrosine, despite the adjacent carboxylate side chain. A similar situation has been uncovered in the enzyme ketosteroid isomerase (*12*), in which the Tyr Y8a/8b bands fail to shift down despite strong H-bond donation from Tyr 14 to the substrate carbonyl group. The reason is that the donor H-bond is compensated by an acceptor H-bond from the nearby Asp 99 side chain (*13*). A similar compensation must be operative for Tyr α42 in Hb. Candidates for the compensating positive polarization are the Asp β94 backbone NH group, and the nearby side chain of Arg β40.

EXPERIMENTAL PROCEDURES

The recombinant hemoglobin (rHb) expression plasmid pHE2 (*14*) containing synthetic α- and β-globin genes and *E. coli* methionine aminopeptidase (MAP) was kindly supplied by Professor Chien Ho. To efficiently incorporate isotopically labeled amino acids into the protein, the plasmid was transformed into host cell *E. coli* DL39 (DE3) which is auxotrophic for six amino acids: Tyr, Asp, Phe, Leu, Ile, and Val (*15*). Natural-abundance rHb was expressed by growing the bacteria in Luria−Bertani (LB) medium. Expression was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG, Sigma) to a concentration of 50 mg/mL when the cell density reached $OD_{600} = 1$. At the time of induction, the culture was supplemented with hemin (20 mg/L, Sigma). Six hours after induction, the cells were harvested by centrifugation and then stored frozen at 77 K until needed for purification.

To produce ^{13}C-labeled hemoglobin, the host cell DL39-(DE3) was grown in a defined M9 medium supplemented with 19 amino acids. [4-^{13}C]-Asp was supplied at a minimal level for sufficient cell growth, which was about 25% of the concentration normally used in the defined media. Specifically, the medium contained 0.10 g of L-[4-^{13}C]-Asp (98%, purchased from Cambridge Isotope Laboratories) and 17 unlabeled L-amino acids (0.50 g of Ala, 0.40 g of Arg, 0.05 g of Cys, 0.40 g of Gln, 0.65 g of Glu, 0.55 g of Gly, 0.10 g of His, 0.23 g of Ile, 0.23 g of Leu, 0.42 g of LysHCl, 0.25 g of Met, 0.13 g of Phe, 0.10 g of Pro, 2.10 g of Ser, 0.23 g of Thr, 0.17 g of Tyr, and 0.23 g of Val), as well as 0.50 g of adenine, 0.65 g of guanosine, 0.20 g of thymine, 0.50 g of uracil, 0.20 g of cytosine, 1.50 g of sodium acetate, 1.50 g of succinic acid, 0.50 g of NH_4Cl, 0.85 g of NaOH, and 10.5 g of K_2HPO_4 per 950 mL of water. (Note: Asn was left out since its presence made host DL39 grow without Asp, presumably due to its partial conversion to Asp). After autoclaving, 50 mL of 20% glucose, 2 mL of 1 M $MgSO_4$, 1 mL of 0.01 M $FeCl_3$, 0.2 mL of 0.1 M $CaCl_2$, and 2 mg $ZnCl_2$, 2 mg of $MnCl_2$, 50 mg of thiamine, 50 mg of Trp, and 50 mg of ampicillin were added under sterile conditions.

The purification procedure is described elsewhere (*16*). The FTIR difference spectra were taken as reported previously (*9*). The mass spectra were provided by the Mass Spectrometry Facility, School of Pharmacy, University of California, San Francisco, using electrospray mass spectrometry (NIH NCRR BRTP RR01614).

Table 1: Masses (Daltons) of [4-^{13}C−Asp]-rHb, NA rHb, and Hb A[a]

	α chain		β chain	
	calculated	experimental	calculated	experimental
Hb A	15126.4	15125.0 ± 0.5	15867.2	15865.9 ± 0.9
NA rHb	15126.4	15127.2 ± 0.9	15867.2	15868.3 ± 3.3
[4-^{13}C]-Asp rHb	15138.4	15137.7 ± 0.7	15880.2	15879.0 ± 9.6

[a] The mass spectra were provided by the Mass Spectrometry Facility, School of Pharmacy, University of California, San Francisco, using electrospray mass spectrometry.

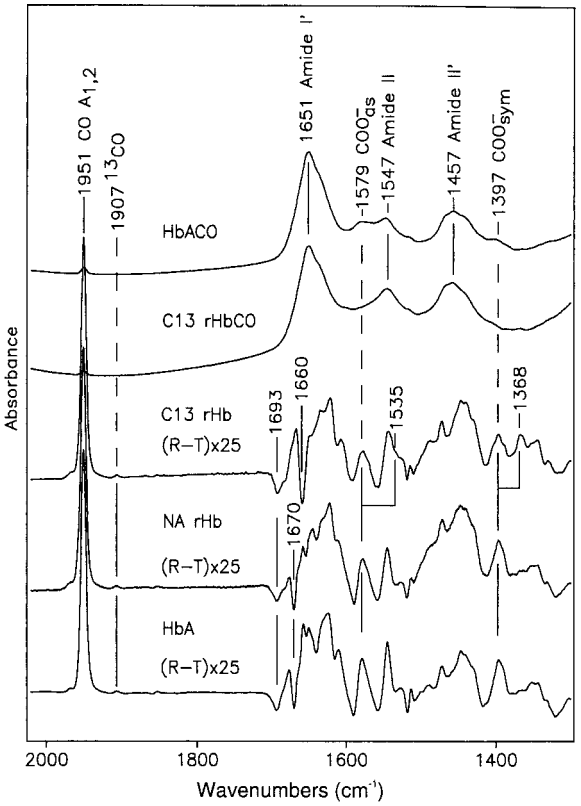

FIGURE 1: FTIR spectra and difference spectra of [4-^{13}C]-Asp-labeled rHb and natural abundance rHb at pD 7.4. Also shown are the spectra of Hb A for comparison purpose. The parent spectra are against N_2 as background, and the difference spectra (labeled as R−T) are multiplied by a factor of 25 for clarity.

RESULTS

Recombinant human Hb was expressed in the DL39 auxotroph of *E. coli* in a medium with all amino acids except aspartate and asparagine; the medium was supplemented with L-[4-^{13}C]-Asp. This auxotroph is deficient in the synthesis of Asp, Tyr, Phe, Leu, and Val (*15*). However, it was found to be capable of interconverting Asp and Asn residues. To avoid dilution of the isotope label, Asn was omitted from the medium. Consequently, the Asn as well as the Asp residues contained ^{13}C label. There are eight Asp and four Asn residues in the α chains, and seven Asp and six Asn residues in the β chain, so that the expected mass increase is 12 daltons for the α chains and 13 daltons for the β chains. Electrospray mass spectrometer data were within experimental error of the expected masses (Table 1).

The difference FTIR spectrum between HbCO and deoxy-Hb is the same as previously reported (*9*), and contains a well-defined negative band at 1693 cm^{-1} (Figure 1). The spectrum is unaltered for natural-abundance recombinant

hemoglobin (NA rHb), establishing native character for the bacterially expressed protein. When the recombinant Hb is labeled with [4-^{13}C]-aspartate, there is no shift in the 1693 cm^{-1} band, although a ~40 cm^{-1} shift would have been expected for a (C═O)OH stretch (*10*). Thus, the difference band cannot arise from an Asp residue. Neither can it arise from an Asn carboxamide side chain, since the Asn residues are also labeled in the experiment.

We searched the region near 1700 cm^{-1} carefully, and found no evidence for another R−T difference band that might arise from (C═O)OH. On the basis of recently published FTIR spectra of another heme protein, cytochrome *bo*$_3$ (*11*), we estimate that a single protonated carboxylate should have produced a difference band of about 3−5 times the amplitude of the 1907 cm^{-1} band, the ^{13}CO satellite (1.08% natural abundance) of the heme-bound CO band (1951 cm^{-1}). This band is clearly observed in the difference spectrum (Figure 1). Thus, the FTIR evidence for Asp β99 protonation is negative.

What is the origin of the 1693 cm^{-1} band? If it is a (C═O)OH mode, then the available candidates are the glutamate side chains, the carboxylate chain termini, and the heme propionate substituents. However, the heme propionates are exposed to solvent, as are all the Glu residues, and the C-termini are involved in salt bridges as anions. Thus, a (C═O)OH assignment seems unlikely. Instead, we tentatively assign the 1693 cm^{-1} band to an amide C═O stretch (amide I). Amide I frequencies are generally lower than 1690 cm^{-1} (*17*), but a higher value is not impossible if H-bonding to the C═O is weak or absent, especially for peptide turns. The 1693 cm^{-1} band could arise from one or several amide groups, either in the backbone or else the carboxamide side chain of a glutamine (but not asparagine), which loses its H-bond in the T state. These amides must be perturbed in Hb Kempsey, which lacks the 1693 cm^{-1} band (*9*). However, Hb Kempsey does have a negative R-minus-T difference band at 1680 cm^{-1}, which might be a shifted position for the same amide groups, if they become more solvent-exposed. Although IHP stabilizes the T state for Hb Kempsey, as it does in native Hb A, it is known that the Kempsey $\alpha_1\beta_2$ interface remains perturbed even in the presence of IHP (*18*).

Although the 1693 cm^{-1} band is unshifted, one does see isotope-sensitive features in the FTIR difference spectra. Weak difference bands at 1579 and 1397 cm^{-1} are partially replaced in the ^{13}C-labeled spectra by downshifted new features at 1535 and 1368 cm^{-1} (Figure 1). These are the correct frequencies for carboxylate anion asymmetric and symmetric stretches (*19*). We note that the bands at 1579 and 1397 cm^{-1} are quite strong in the parent spectra (top trace in Figure 1) because there are 15 Asp residues per dimer, but most of the intensity cancels in the difference spectrum. The weak remnant intensity may reflect altered environments for one or more aspartate carboxylate anions between the R and T states. There are other contributions to the difference signal at these frequencies, so it is imposible to tell whether the Asp frequencies shift between R and T states.

There is also a negative band at 1670 cm^{-1} in the natural-abundance sample and at 1660 cm^{-1} in the ^{13}C-labeled sample. We considered the possibility that this arises from an asparagine carboxamide, but the apparent downshift is too small for amide I, which is expected to shift ~40 cm^{-1} upon ^{13}C substitution. This difference feature is in the very crowded amide I region of the spectrum, and may result from compensating shifts in more than one parent IR band.

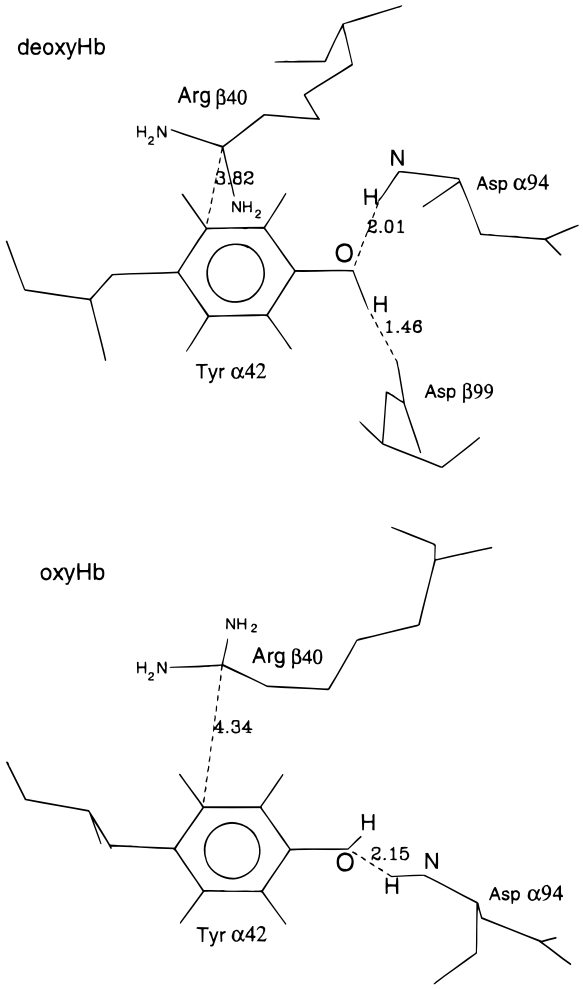

FIGURE 2: Proposed H-bonding environment of Tyr α42 in deoxyHb and in oxyHb based on the X-ray coordinates (*25*, *26*) in the Brookhaven database.

DISCUSSION

As pointed out earlier (*9*), the protonation state of Asp β99 is not obvious by inspection. In the T structure, it is inaccessible to solvent (*5*), but on the other hand it is part of a network of H-bonds (*9*) that could stabilize a carboxylate anion. If Asp β99 were protonated in the T state, it would contribute two Bohr protons per tetramer. About two protons are in fact released at pH 7.4, but these are already accounted for by other residues [(*20*) and references cited therein], and if two more Asp β99 protons were released, one would have to postulate a compensating uptake of two protons in the R state, for which there is currently no evidence. In view of the present FTIR results, we conclude that the Asp β99 side chain is ionized in the T as well as the R state.

However, we are left with an apparent conundrum with respect to the UVRR bands Y8a/8b and Y7a′, which shift in directions opposite to those expected if tyrosine is an H-bond donor (*5*, *7*). Although there are six tyrosine residues per Hb dimer, an isotope hybrid experiment has identified the α chains as the source of the Y8a upshift (*16*). Of the three α chain Tyr residues, only Tyr α42 experiences a significant

T−R change in its H-bond status. Moreover, the Y8a upshift is abolished in the double mutant Yα42D/Dβ99N (*21*), although the T state is maintained. Thus, we can say with some confidence that the expected downshift for Tyr α42 is overridden by a countervailing influence.

There is precedent for such an effect in the enzyme Δ^5-3-ketosteroid isomerase (KSI), in which the Tyr 14 side chain acts as a general acid via H-bond donation to the substrate carbonyl group (*22*). This proton gives a downfield NMR resonance at 11.6 ppm relative to H_2O (*13*), similar to the 9.4 ppm resonance of the Tyr α42−Asp β99 proton in Hb (*23*). Yet the Y8a/8b bands of Tyr 14 are not downshifted upon substrate binding, and it was proposed that the expected downshift is overridden by a compensating H-bond to the Tyr 14 OH from a nearby donor (*12*, *24*). This donor has now been identified as the Asp 99 side chain of KSI, which has a pK_a of 9.5 (*13*).

A search for compensating H-bonds in the Hb structures (*25*, *26*) reveals that the Asp β94 backbone NH donates an H-bond to the Tyr OH; H···O = 2.01 Å, ∠N−H···O = 168° in the T state (Figure 2). This H-bond is still present in the R state, but appears to weaken (H···O = 2.15 Å, ∠N−H···O = 154°), consistent with compensation of the Tyr α42−Aspβ99 H-bond in the T state. However, this backbone H-bond is clearly weaker than the aspartic acid H-bond in KSI, and is unlikely to account for the UVRR shift reversals. We suggest that the H-bond compensation is strongly augmented in deoxyHb by polarization from the nearby Arg β40 residue (Figure 2). The guanidium C atom is 4.34 Å from the nearest ring C atom of Tyr α42 in the R state, but is 0.5 Å closer in the T state. This displacement moves a positive charge toward Tyr α42, and is expected to shift the tyrosine modes in the same direction as H-bond acceptance by the OH group. Moreover, the closest negative charge is on Glu β43, whose carboxylate is 6.3 Å from the Arg β40 guanidium ion in the R state, but 8.7 Å in the T state structure (*25*, *26*). Thus, the Arg β40 positive charge is essentially uncompensated in the T state and should produce a substantial field at Tyr α42. The polarization by the backbone NH and by Arg β40 may be of functional significance, since it should contribute to the strength of the Tyr α42−Asp β99 H-bond, and thereby to the T state stability.

In summary, we conclude that Asp β99 is deprotonated, and accepts an H-bond from Tyr α42 in the T state, as has been supposed (*1*). In addition, this H-bond is strengthened by compensating H-bond donation from the Asp β94 backbone NH, and by displacement of the Arg β40 side chain toward Tyr α42. The resulting positive polarization overrides the expected H-bond donor shifts of the Tyr α42 ring modes in the UVRR spectrum.

REFERENCES

1. Perutz, M. F., and Ten Eyck, L. F. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 295−310.
2. Imai, K., Fushitani, K., Miyazaki, G., Ishimori, K., Kitagawa, T., Wada, Y., Morimoto, H., Morishima, I., Shih, D. T., and Tame, J. (1991) *J. Mol. Biol. 218*, 769−778.
3. Yanase, H., Cahill, S., Martin de Llano, J. J., Manning, L. R., Schneider, K., Chait, B. T., Vandegriff, K. D., Winslow, R. M., and Manning, J. M. (1994) *Protein Sci. 3*, 1213−1223.
4. Bunn, H. F., Wohl, R. C., Bradley, T. B., Cooley, M., and Gibson, Q. (1974) *J. Biol. Chem. 249*, 7402−7409.
5. Rodgers, K. R., Su, C., Subramanian, S., and Spiro, T. G. (1992) *J. Am. Chem. Soc. 114*, 3697−3709.
6. Hildebrandt, P. G., Copeland, R. A., Spiro, T. G., Otlewski, J., Laskowshi, M., and Prendergast, F. G. (1988) *Biochemistry 27*, 5426−5433.
7. Zhao, X., and Spiro, T. G. (1998) *J. Raman Spectrosc. 29*, 49−55.
8. Takeuchi, H., Watanabe, N., Satoh, U., and Harada, I. (1989) *J. Raman Spectrosc. 20*, 233−237.
9. Gregoriou, V. G., Jayaraman, V., Hu, X., and Spiro, T. G. (1995) *Biochemistry 34*, 6876−6882.
10. Engelhard, M., Gerwert, K., Hess, B., Kreutz, W., and Siebert, F. (1985) *Biochemistry 24*, 400−407.
11. Puustinen, A., Bailey, J. A., Dyer, R. B., Mecklenburg, S. L., Wikstrom, M., and Woodruff, W. (1997) *Biochemistry 36*, 13195−13200.
12. Austin, J. C., Kuliopulos, A., Mildvan, A. S., and Spiro, T. G. (1992) *Protein Sci. 1*, 259−270.
13. Zhao, Q., Abeygunawardana, C., Gittis, A. G., and Mildvan, A. S. (1997) *Biochemistry 36*, 14616−14626.
14. Shen, T.-J., Ho, N. T., Simplaneaun, V., Zou, M., Green, B. N., Tam, M. F., and Ho, C. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8108−8112.
15. LeMaster, D. M., and Richards, F. M. (1988) *Biochemistry 27*, 142−150.
16. Hu, X., and Spiro, T. G. (1997) *Biochemistry 36*, 15701−15712.
17. Byler, D. M., and Susi, H. (1986) *Biopolymers 25*, 469−487.
18. Hu, X., Rodgers, K. R., Mukherji, I., and Spiro, T. G. (1998) (submitted for publication).
19. Hammaker, R. M., and Walters, J. P. (1964) *Spectrochim. Acta 20*, 1311−1317.
20. Sun, D. P., Zou, M., Ho, N. T., and Ho, C. (1997) *Biochemistry 36*, 6663−6673.
21. Huang, S., Peterson, E. C., Ho, C., and Friedman, J. M. (1997) *Biochemistry 36*, 6197−6206.
22. Kuliopulos, A., Mildvan, A. S., Shortle, D., and Tatalay, P. (1989) *Biochemistry 28*, 149−159.
23. Fung, L. W.-M., and Ho, C. (1975) *Biochemistry 14*, 2526−2535.
24. Austin, J. C., Zhao, Q., Jordan, T., Talalay, P., Mildvan, A. S., and Spiro, T. G. (1995) *Biochemistry 34*, 4441−4447.
25. Fermi, G., Perutz, M. F., Shaanan, B., and Fourme, R. (1984) *J. Mol. Biol. 175*, 159−174.
26. Shaanan, B. (1983) *J. Mol. Biol. 171*, 31−59.

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