REFERENCES
Adams, M. H. (1959) in *Bacteriophage*, pp 450–454, Interscience, New York.
Barton, J. K., & Raphael, A. L. (1984) *J. Am. Chem. Soc. 106*, 2466–2468.
Bryant, J. L., Jr., & King, J. (1984) *J. Mol. Biol. 180*, 837–863.
Cai, J.-C., & Hutchinson, C. R. (1983) in *The Alkaloids: Chemistry and Pharmacology* (Brossi, A., Ed.) Vol. 21, pp 101–137, Academic Press, New York.
Carteright, I. L., & Elgin, S. C. R. (1982) *Nucleic Acids Res. 10*, 5835–5852.
Falk, K.-E., Freeman, H., Jansson, T., Malmström, B., & Vanngard, T. (1967) *J. Am. Chem. Soc. 89*, 6071–6077.
Fukada, A., & Sinsheimer, R. L. (1976) *J. Virol. 17*, 776–787.
Fukada, M. (1980) *J. Biochem. (Tokyo) 87*, 1089–1096.
Guthrie, G. D., & Sinsheimer, R. L. (1963) *Biochim. Biophys. Acta 72*, 290–297.
Horwitz, S. B., & Horwitz, M. S. (1971) *Biochem. Biophys. Res. Commun. 45*, 723–727.
Jain, S. C., Bhandary, K. K., & Sobell, H. M. (1979) *J. Mol. Biol. 135*, 813–840.
Lown, J. W., & Chen, H.-H. (1980) *Biochem. Pharmacol. 29*, 905–915.
Marshall, L. E., Graham, D. R., Reich, K. A., & Sigman, D. S. (1981) *Biochemistry 20*, 244–250.
Marshall, L. E., Reich, K. A., Graham, D. R., & Sigman, D. S. (1982) *J. Biol. Chem. 257*, 12121–12128.
Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–560.
Misra, R. P., Muench, K. F., & Humayun, M. Z. (1983) *Biochemistry 22*, 3351–3359.
Moore, H. W. (1977) *Science (Washington, D.C.) 197*, 527–532.
Sigman, D. S., Graham, D. R., D'Aurora, V. D., & Stern, A. M. (1979) *J. Biol. Chem. 254*, 12269–12272.
Sugiura, Y., & Suzuki, T. (1982) *J. Biol. Chem. 257*, 10544–10546.
Sugiura, Y., Kuwahara, J., & Suzuki, T. (1984) *Biochim. Biophys. Acta 782*, 254–261.
Sugiura, Y., Takita, T., & Umezawa, H. (1985) in *Metal Ions in Biological Systems* (Sigel, H., Ed). Vol. 19, pp 81–108, Marcel Dekker, New York.
Suzuki, T., Kuwahara, J., Goto, M., & Sugiura, Y. (1985a) *Biochim. Biophys. Acta 824*, 330–335.
Suzuki, T., Kuwahara, J., & Sugiura, Y. (1985b) *Biochemistry 24*, 4719–4721.
Wall, M. E., & Wani, M. C. (1980) in *Anticancer Agents Based on Natural Product Models* (Cassady, J. M., & Douros, J. D., Eds.) p 417, Academic Press, New York.
Wong, A., Huang, C.-H., & Crooke, S. T. (1984) *Biochemistry 23*, 2946–2952.

Proton Nuclear Magnetic Resonance Investigation of the Spin-State Equilibrium of the α and β Subunits in Intact Azidomethemoglobin

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ABSTRACT: The hyperfine-shifted proton NMR spectra of human azidomethemoglobin were examined at 300 MHz in the 2–60 °C range. From analysis of the temperature-dependent heme methyl shifts, the thermal spin-state equilibria of the α and β subunits were independently analyzed in the intact tetramer. The thermodynamic values of the spin equilibrium of the α and β subunits were comparable, suggesting that the spin equilibrium properties of the constituent subunits are similar to each other. Examination of the azidomethemoglobins reconstituted with deutero- or mesohemin further shows that the α and β subunit difference is still small in these hemoglobins probably due to the smallness of the steric and electronic difference of the heme 2,4-substituents of the examined porphyrins. The similarity of the spin equilibrium profiles of the subunits indicates that the strain imposed from the globin to the heme iron is of comparable magnitude for the α and β subunits within the azidomethemoglobins.

Considerable attention has been paid to the spin state of hemoglobin because a change of iron spin is involved in ligand binding to deoxyhemoglobin. In an effort to investigate the structural alterations by ligand binding, the magnetic properties of hemoglobin have been measured for ferrous (Cerdonio et al., 1985; Savicki et al., 1984) and ferric (Beetlestone & George, 1964; Iizuka & Yonetani, 1970) derivatives. Among the methemoglobin derivatives, azidomethemoglobin, which is in a thermal spin-state equilibrium, has been extensively characterized with Mössbauer (Winter et al., 1972), infrared (McCoy & Caughey, 1970; Alben & Fager, 1972), resonance Raman (Scholler & Hoffman, 1979; Cho et al., 1981), electron spin resonance (Scholler & Hoffman, 1979), and visible absorption (Neya et al., 1983) spectroscopies and magnetic susceptibility measurements (Beetlestone & George, 1964; Iizuka & Kotani, 1969; Philo & Dreyer, 1985; Messana et al., 1978). Although these physical methods reveal the bulk magnetic properties, they do not discern possible differences between the α and β subunits. Observation of the isolated subunits or valency hybrid hemoglobin may provide information for the individual chain, but the iron spin state may be perturbed due to the structural alteration accompanying subunit separation or the heme–heme interaction between the ferrous and ferric subunits.

The proton NMR spectrum of azidomethemoglobin resolves the α- and β-heme methyl protons (Davis et al., 1969; Iizuka

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& Morishima, 1974; Morishima et al., 1978; Neya & Morishima, 1980). In the previous analysis of the temperature-dependent heme methyl protons, however, the same thermodynamic values of the spin-state equilibrium, which were obtained from paramagnetic susceptibility measurements (Iizuka & Kotani, 1969), were assumed for the α and β subunits, and the thermodynamic parameters were not distinguished between them (Morishima et al., 1978).

It will be of fundamental interest to analyze the spin state of the constituent subunits within intact tetramer. By taking advantage of proton NMR spectra, which discriminate the α and β subunits of azidomethemoglobin, we attempted an independent determination of the spin equilibrium parameters of the constituent subunits in tetrameric hemoglobin. We applied the NMR analysis also to the azidomethemoglobins reconstituted with deutero- or mesohemin to examine the effect of the heme 2,4-substituents on spin equilibrium properties of the α and β subunits.

Materials and Methods

Chemicals. *N*-Ethylmaleimide, NaN_3 (Nakarai Chemicals, Kyoto, Japan), and Pipes[1] (Dojin Laboratories, Kumamoto, Japan) were commercially available. Deuterium oxide and DSS were obtained from Merck.

Methemoglobin. Human methemoglobin was prepared from whole blood drawn from one of the authors (S.N.) according to the reported method (Neya & Morishima, 1981). The heme concentration of azidomethemoglobin solution was determined by measuring the absorbance at 542 nm [ϵ = 11.4 mM^{-1} cm^{-1} (Perutz et al., 1974)].

Reconstituted Hemoglobin. Apohemoglobin was prepared by the procedure of Asakura (1978) and determined with an extinction coefficient at 280 nm of 12.7 mM^{-1} cm^{-1} per subunit (Scholler et al., 1978). Deutero- and mesohemins were synthesized from protohemin (Sigma, type I) according to the reported methods (Caughey et al., 1966). The purity and identity of the synthesized hemins were checked by the pyridine hemochromogen method (Antonini & Brunori, 1971) and proton NMR spectra of the bis(cyanide) derivatives (La Mar et al., 1978).

Combination of globin with deutero- or mesohemin was performed as described by Asakura (1978). The crude reconstituted methemoglobin was dialyzed against several changes of 10 mM Pipes, pH 6.0 and loaded on a CM-cellulose (Whatman, CM-52) column equilibrated with the same buffer. The cellulose column was washed with 10 mM Pipes, pH 6.0, and the absorbed methemoglobin was eluted with 0.1 M Pipes, pD 7.0, in D_2O for solvent exchange from H_2O to D_2O. The heme concentration of the reconstituted hemoglobin was determined with the known extinction coefficients (Sugita & Yoneyama, 1971). The azide complex was prepared by adding an appropriate amount of NaN_3 to aquomethemoglobin solutions.

Spectroscopic Measurements. Electronic absorption spectra were obtained by using a Shimadzu MPS-2000 spectrophotometer.

Proton NMR spectra at 300 MHz were recorded on a Varian XL-300 spectrometer with a temperature variation unit. Probe temperature was determined by monitoring the chemical shift difference of ethylene glycol before and after measurement, accurate to ±0.1 °C. Typical NMR spectra consisted of 5000 pulses and were collected by using 8K data points over a 20-kHz spectral width. The residual water signal was suppressed by a presaturation pulse from the decoupler. Signal to noise ratio was improved by exponential apodization which introduced 5-Hz line broadening. Chemical shifts (±0.01 ppm) were referenced to internal DSS with a positive sign for downfield resonances. Sample volume of the NMR sample was 0.4 mL, and heme concentration was about 4 mM. The pD value of the NMR sample was the direct reading of a pH meter, Toko-Kagaku Model TP-1000, equipped with a combination electrode, Toko-Kagaku Model CE-105.

Data Analysis. Computer-assisted data analysis was carried out on a Sharp MZ-2000 microcomputer.

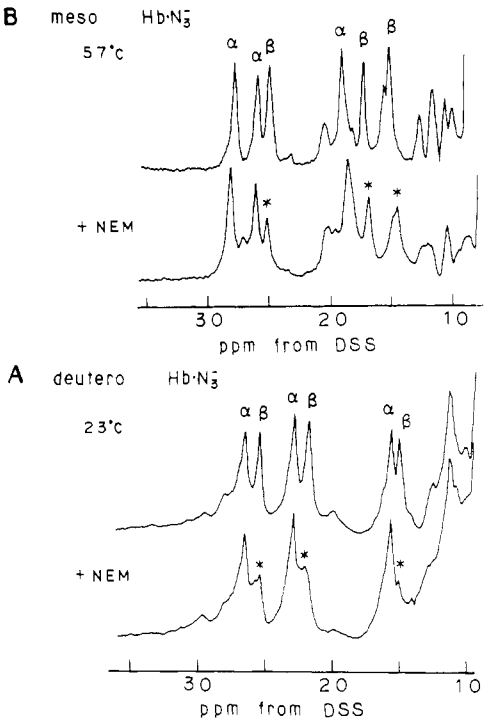

FIGURE 1: Assignment of the α- and β-heme methyl peaks of the proton NMR spectra of the azidomethemoglobins: (A) deuterohemin hemoglobin at 23 °C and (B) mesohemin hemoglobin at 57 °C in 0.1 M Pipes, pD 7.0, containing 50 mM NaN_3. The heme methyl peaks that were affected by *N*-ethylmaleimide (NEM) were labeled with asterisks and assigned to the β-heme methyl protons. The NEM/heme ratio was 0.2.

Results

Assignment of the α- and β-Heme Methyl Peaks. Recent proton NMR studies revealed that when globin is reconstituted with hemin, the initial complex formed is a 1:1 mixture of heme orientational isomers (La Mar et al., 1983a,b, 1984). One is more stable than the other, and the stable conformer dominates with time. In view of these results, it is very likely that a small fraction of a less stable conformer still remains in our preparations. From the NMR peak intensity comparison of the heme methyl with other minor resonances at 60 °C, the fraction of the major conformer was estimated to be more than 0.8 for the presently reconstituted deutero- and mesohemin azidomethemoglobins. Then, we are concerned only with the stable conformer which exhibits the dominant heme methyl resonances. The heme methyl peaks are readily distinguished from other peaks because of their relatively high signal intensity. The assignment of the heme methyl protons to the α and β subunits is essential to monitor the constituent subunits independently in the intact tetramer. Figure 1A shows the proton NMR spectra of the azidomethemoglobin

[1] Abbreviations: Pipes, 2,2′-piperazine-1,4-diylbis(ethanesulfonic acid); DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; Hb, hemoglobin.

reconstituted with deuterohemin. The spectrum is similar to that of native azidomethemoglobin (Davis et al., 1969; Iizuka & Morishima, 1974; Morishima et al., 1978; Neya & Morishima, 1981). For the assignment of the α- and β-heme methyl peaks, we examined *N*-ethylmaleimide binding to the hemoglobin. We have reported that *N*-ethylmaleimide affects only the β-heme methyl peaks of methemoglobin derivatives due to selective binding of the sulfhydryl reagent to β cysteine-93 (Neya & Morishima, 1980). Upon addition of *N*-ethylmaleimide, the intensity of the heme methyl peaks that are labeled with asterisks significantly decreased. The affected and unaffected heme methyl peaks were then assigned to the β and α subunits, respectively.

The *N*-ethylmaleimide binding to the mesohemin hemoglobin was examined at 57 °C because the room temperature spectrum is not clearly resolved. Figure 1B shows that the NMR spectrum at 57 °C is comparable with those of proto- and deuterohemin hemoglobins recorded at comparable temperature (vide infra). In the mesohemin hemoglobin spectrum, the heme methyl peaks that were selectively affected by *N*-ethylmaleimide were assigned to the β subunit resonances.

Assignment of the α- and β-heme methyl peaks of native azidomethemoglobin has been reported (Davis et al., 1969; Neya & Morishima, 1980).

Temperature Dependence of the Heme Methyl Shifts. Figure 2 shows the proton NMR spectra of azidomethemoglobins containing proto-, deutero-, or mesohemin recorded in the 2–60 °C range. The chemical shifts of the heme methyl protons are temperature-dependent; some of the peaks show upfield shift and others downfield shift with increasing temperature. Figure 3 illustrates the temperature dependence of the heme methyl shifts. The Curie plot of native azidomethemoglobin is essentially the same as those reported previously (Iizuka & Morishima, 1974; Morishima et al., 1978) except that the present data are examined in more detail at higher magnetic field. It is to be noted that all the heme methyl shifts exhibit deviation from the Curie law, which predicts linear temperature dependence of the hyperfine-shifted resonance shift on the reciprocal of absolute temperature (Wüthrich, 1970). In addition, the α_3- and β_3-heme methyl peaks in Figure 3 show slopes opposite in sign to those expected from the Curie law. These deviations from the Curie law are indicative of thermal spin-state equilibrium between the high- and low-spin states (Iizuka & Morishima, 1974; Morishima et al., 1978; La Mar et al., 1983c).

Analysis of the Heme Methyl Shifts. The observed heme methyl shift δ consists of diamagnetic and isotropic contributions (Jesson, 1973; Wüthrich, 1970). The diamagnetic shift may be estimated from the NMR spectrum of diamagnetic metalloporphyrins. Wüthrich (1970) reported a 3.6 ppm shift for the heme methyl protons of the Zn(II) complexes of proto-, deutero-, and mesohemins. In spin equilibrium system, the isotropic shift arises from overlapping contributions of the high- and low-spin isomers and is formulated as $\delta - 3.6 = \alpha\delta_l + (1 - \alpha)\delta_h$, where δ_h and δ_l are the isotropic shifts of the high- and low-spin isomers, respectively, and α is the low-spin fraction. In the case of axial symmetry with a single populated spin level with a negligible second-order Zeeman interaction, the isotropic shift is reduced to a form that obeys the Curie law (Jesson, 1973). Then we obtain

$$\delta - 3.6 = \alpha(L/T) + (1 - \alpha)(H/T) \qquad (1)$$

where L and H are temperature-independent constants and T is absolute temperature. The temperature dependence of the spin equilibrium constant, K = (low spin)/(high spin) = $\alpha/(1 - \alpha) = [H - T(\delta - 3.6)]/[T(\delta - 3.6) - L]$, is described

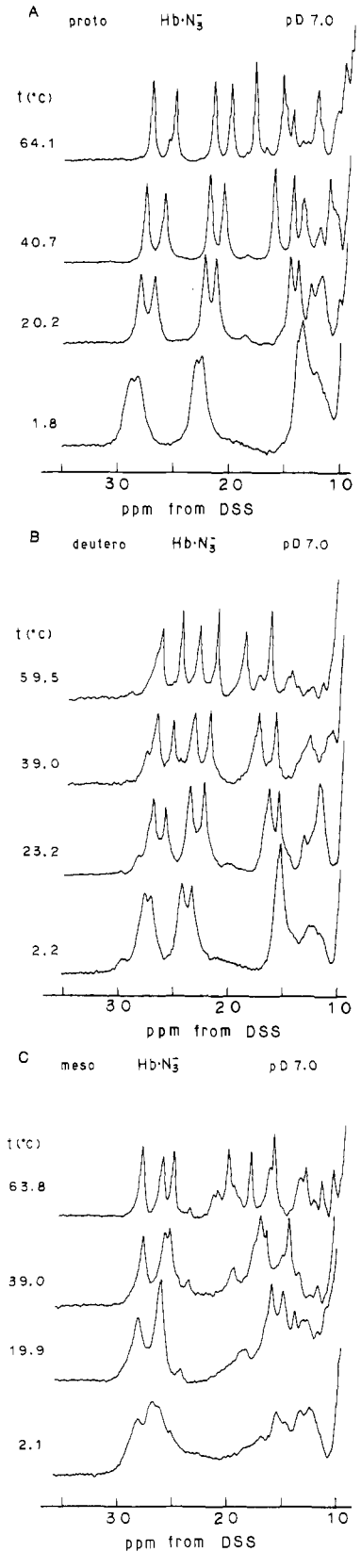

FIGURE 2: Proton NMR spectra of human azidomethemoglobin containing (A) proto-, (B) deutero-, or (C) mesohemin in 0.1 M Pipes, pD 7.0, plus 50 mM NaN_3 at the indicated temperatures.

by the van't Hoff equation, $\ln K = -\Delta H/RT + \Delta S/R$, where R is the gas constant. With these relations, the van't Hoff equation may be written as

$$\ln \frac{H - T(\delta - 3.6)}{T(\delta - 3.6) - L} = -\frac{\Delta H}{R}\frac{1}{T} + \frac{\Delta S}{R} \qquad (2)$$

Table I: Thermodynamic Values of the Spin Equilibrium of Human Azidomethemoglobin Subunits in Intact Tetramer[a]

hemin	subunit	ΔH (cal/mol)	ΔS (eu)	T_c[b] (K)	low-spin fraction at 20 °C
proto	α	−4980 ± 200	−13.3 ± 0.7	374 ± 35	0.86 ± 0.08
	β	−5060 ± 210	−12.5 ± 0.6	405 ± 36	0.90 ± 0.05
deutero	α	−5540 ± 200	−15.2 ± 0.6	364 ± 28	0.85 ± 0.07
	β	−5610 ± 270	−14.5 ± 0.9	387 ± 43	0.88 ± 0.08
meso	α	−5060 ± 120	−13.1 ± 0.7	386 ± 30	0.88 ± 0.06
	β	−5440 ± 150	−13.5 ± 0.5	403 ± 26	0.91 ± 0.04

[a] In 0.1 M Pipes, pD 7.0, containing 50 mM NaN_3. [b] Compensation temperature where the high- and low-spin fractions are the same.

Table II: H and L Values in ppm·K Used for the Chemical Shift Calculation of the Heme Methyl Protons

hemin	methyl peak	H	L	methyl peak	H	L
proto	α_1	10650	6600	β_1	9400	6550
	α_2	8150	5000	β_2	7300	4950
	α_3	10450	2000	β_3	9200	2400
deutero	α_1	9600	6400	β_1	8400	6350
	α_2	7900	5500	β_2	7150	5300
	α_3	9400	2700	β_3	8800	3000
meso	α_1	12550	6400	β_1	10450	6200
	α_2	11650	5850	β_2	11400	2900
	α_3	15600	1700	β_3	10200	2350

The "best" values of H and L are defined as those that give a linear van't Hoff equation. The H and L values were varied between 0 and 20000 ppm·K at 50 ppm·K intervals, and all their possible combinations were checked to fit the observed δ and T values by least squares to the van't Hoff equation with the aid of a microcomputer. For this purpose, we used the computer program that was originally employed for the analysis of the thermochromism (Neya et al., 1985a). When we analyzed the anormal temperature dependence of the α_3 (β_3) heme methyl shift of the mesohemin hemoglobin in Figure 3, the best linear correlation was obtained at H = 15600 (10200) and L = 1700 (2350) ppm·K with a correlation coefficient (Mortimer, 1981) r = 0.9980 (0.9996). Analysis of the α_3- and β_3-heme methyl shifts of the proto- and deuterohemin hemoglobins yielded comparable values with $r >$ 0.9980. In Tables I and II are summarized the parameters obtained from the analysis. The solid curves in Figure 3 represent the least-squares fit calculated with eq 1 with the parameters in Tables I and II.

Discussion

Spin-State Equilibrium in the α and β Subunits. Proton NMR observation of azidomethemoglobin allows independent examination of the spin equilibria of the α and β subunits in the intact tetramer. The simulated shifts of the heme methyl peaks in Figure 3 reproduce well the observed values, suggesting the validity of the present analysis. The averaged thermodynamic values between the α and β subunits, ΔH = −5020 cal/mol and ΔS = −12.9 eu, compare well with the spin equilibrium parameters obtained from the bulk paramagnetic susceptibility (Iizuka & Kotani, 1969), infrared (Alben & Fager, 1972), and visible absorption (Neya et al., 1983) measurements.

In considering possible differences of the α and β subunits in spin equilibrium, Perutz et al. (1978) analyzed the infrared azide stretching bands of azidomethemoglobin A. From the infrared spectral comparison with hemoglobin M Milwaukee, they suggested that the high-spin fractions are ~0.0 and ~0.2 in the α and β subunits, respectively, and that the high-spin component of azidomethemoglobin exclusively comes from the β subunits. In contrast to these suggestions, the proton NMR results in Figure 2 qualitatively show that both of the α- and β-heme methyl peaks exhibit anomalous temperature dependence and the anomaly is prominent in the α_3 peaks. Quantitative analysis of the NMR results indicates that the high-spin fractions are 0.14 ± 0.08 in the α subunits and 0.10 ± 0.05 in the β subunits at 20 °C. This suggests that the high-spin fraction is almost equally distributed between the α and β subunits. The visible spectral observation of spin equilibrium in azidomethemoglobin shows that spectral transitions with clear isosbestic points are observed (Neya et al., 1983), in agreement with the lack of appreciable difference of the spin equilibrium properties of the α and β subunits.

The model complex study of azidomethemoprotein has suggested that the spin equilibrium mechanisms of hemin–azide–imidazole systems inside and outside of the heme pocket are essentially identical and that the position of spin equilibrium is primarily determined by the ligand field strength of the axial base trans to the azide (Neya et al., 1985b). In view of the model complex study and the present spin equilibrium analysis of the α and β subunits, the magnitude of the Fe–N-(His F8) interaction is almost identical between the α and β subunits within intact azidomethemoglobin.

Reconstituted Hemoglobin. As shown in Table I, the thermodynamic parameters of the methemoglobin reconstituted with deutero- or mesohemin[2] are similar to those of protohemin hemoglobin. The van der Waals volume of the heme 2,4-substituents of deutero-, meso-, or protohemin is 12.9, 110.6, or 115.4 Å^3, respectively (Verloop et al., 1976). The changes in the van der Waals volume of the heme substituents must affect the globin–porphyrin interactions in the heme pocket. Despite this expectation, there is no direct dependence of ΔH and ΔS on the molecular volume of the substituents.

The thermodynamic parameters of azidohemoprotein are thought to include the intrinsic values of the prosthetic group and the values of all conformational changes of globin associated with the spin-state transition. The model complex study of azidohemoprotein suggests that globin has only a small effect on the spin equilibrium of azidometmyoglobin (Neya et al., 1985b). In azidomethemoglobin, a small effect of globin has been suggested. The insensitivity of the thermodynamic values in Table I to the heme substitution suggests that the changes in van der Waals contacts affect the thermodynamic properties of azidomethemoglobin only slightly, as in the case of azidometmyoglobin. This is comparable with the results of native azidomethemoglobin in Table I, showing that the α and β subunits have similar spin equilibrium profiles although their heme peripheral environments are significantly different

[2] From the paramagnetic susceptibility measurements in a 77–250 K range, Yonetani et al. (1972) have reported ΔH = −3020 cal/mol and ΔS = −7.27 eu for azidomethemoglobin reconstituted with mesohemin. The bulk thermodynamic values are comparable but appreciably less negative than those of the subunits in Table I. The discrepancy between their and our values may arise from the difference in the examined temperature ranges (77–250 K vs. 270–330 K) and the fractions of the heme orientational isomers in the reconstituted mesohemin hemoglobin. Despite the difference in the thermodynamic parameters, the α and β subunits appear to have similar magnetic properties in the stable conformer of mesohemin azidomethemoglobin.

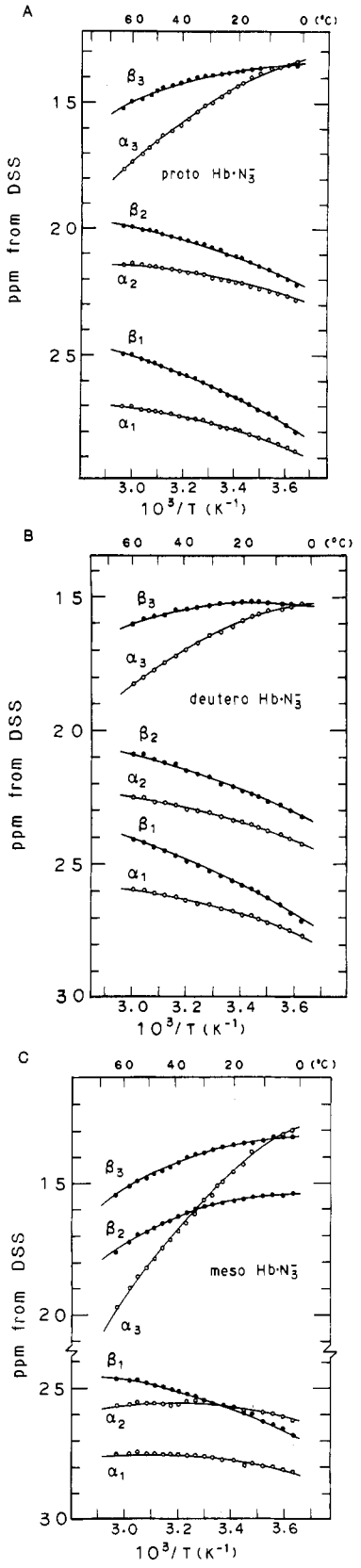

FIGURE 3: Temperature dependence of the α- (O) and β-heme (●) methyl shifts of azidomethemoglobins containing (A) proto-, (B) deutero-, or (C) mesohemin in 0.1 M Pipes, pD 7.0, plus 50 mM NaN_3. The solid curves represent the least-squares fit calculated with the parameters in Tables I and II.

from each other (Ladner et al., 1977; Detherage et al., 1979).

The pK_3 value of porphyrin has been used as a measure of the electronic effect of the heme substituents. Porphyrin with larger pK_3 donates more electron density to heme iron to decrease its charge attraction for axial ligands. This results in weakening of axial ligand field and shifts the spin equilibrium to high spin (Geiger & Scheidt, 1984). The pK_3 values of proto-, deutero-, and mesohemins are 4.8, 5.5, and 5.8, respectively (Falk, 1964). The similarity of the spin equilibrium properties of azidomethemoglobins containing these hemins suggests that the electronic difference is not large enough to manifest the heme difference. Table I further shows that the subunit equivalence is not affected by the heme substitution. The similar spin equilibrium properties of the subunits indicate that the tension from globin to heme iron is almost identical between the α and β subunits within mesohemin- or deuterohemin-containing azidomethemoglobin A.

REFERENCES

Alben, J. C., & Fager, L. Y. (1972) *Biochemistry 11*, 842–847.

Antonini, E., & Brunori, M. (1971) in *Hemoglobin and Myoglobin in Their Reactions with Ligands*, p 10, North-Holland, Amsterdam.

Asakura, T. (1978) *Methods Enzymol. 52*, 447–455.

Beetlestone, J., & George, P. (1964) *Biochemistry 3*, 707–714.

Caughey, W., Alben, J. O., Fujimoto, W., & York, J. L. (1966) *J. Org. Chem. 31*, 2631–2640.

Cerdonio, M., Morante, S., Torresani, D., Vatale, S., DeYoung, A., & Noble, R. W. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 102–103.

Cho, K. C., Remba, R. P., & Fitchen, D. B. (1981) *Biochim. Biophys. Acta 668*, 186–192.

Davis, D. G., Charache, S., & Ho, C. (1969) *Proc. Natl. Acad. Sci. U.S.A. 60*, 1403–1409.

Detherage, J. F., Obendorf, S. K., & Moffat, K. (1979) *J. Mol. Biol. 134*, 419–429.

Falk, J. E. (1964) in *Porphyrins and Metalloporphyrins*, p 28, Elsevier, Amsterdam.

Geiger, D. K., & Scheidt, W. R. (1984) *Inorg. Chem. 23*, 1970–1972.

Iizuka, T., & Kotani, M. (1969) *Biochim. Biophys. Acta 194*, 351–363.

Iizuka, T., & Yonetani, T. (1970) *Adv. Biophys. 1*, 157–182.

Iizuka, T., & Morishima, I. (1974) *Biochim. Biophys. Acta 371*, 1–13.

Jesson, J. P. (1973) in *NMR of Paramagnetic Molecules* (La Mar, G. N., Horrocks, W. D., Holm, R. H., Eds.) pp 1–52, Academic Press, New York.

Ladner, R. C., Heidner, E. J., & Perutz, M. F. (1977) *J. Mol. Biol. 114*, 385–414.

La Mar, G. N., Viscio, D. B., Smith, K. M., Caughey, W. S., & Smith, M. L. (1978) *J. Am. Chem. Soc. 100*, 8085–8092.

La Mar, G. N., Davis, N. L., Parish, D. W., & Smith, K. M. (1983a) *J. Mol. Biol. 168*, 887–896.

La Mar, G. N., de Ropp, J. S., Smith, K. M., Langry, K. C. (1983b) *J. Am. Chem. Soc. 105*, 4576–4580.

La Mar, G. N., Krishnamoorthi, R., Smith, K. M., Gersonde, K., & Sick, H. (1983c) *Biochemistry 22*, 6239–6246.

La Mar, G. N., Toi, H., & Krishnamoorthi, R. (1984) *J. Am. Chem. Soc. 106*, 6395–6401.

McCoy, S., & Caughey, W. S. (1970) *Biochemistry 9*, 2387–2393.

Messana, C., Cerdonio, M., Shenkin, P., Noble, R. W., Fermi, G. F., Perutz, R. N., & Perutz, M. F. (1978) *Biochemistry 17*, 3652–3662.

Morishima, I., Neya, S., Inubushi, T., Yonazawa, T., & Iizuka, T. (1978) *Biochim. Biophys. Acta 534*, 307–316.

Mortimer, R. G. (1981) in *Mathematics for Physical Chemistry*, p 295, Macmillan, New York.

Neya, S., & Morishima, I. (1980) *Biochemistry 19*, 258–265.

Neya, S., & Morishima, I. (1981) *J. Biol. Chem. 256*, 793-798.
Neya, S., Hada, S., & Funasaki, N. (1983) *Biochemistry 22*, 3686-3691.
Neya, S., Hada, S., & Funasaki, N. (1985a) *Biochim. Biophys. Acta 828*, 241-246.
Neya, S., Hada, S., Funasaki, N., Umemura, J., & Takenaka, T. (1985b) *Biochim. Biophys. Acta 827*, 157-163.
Perutz, M. F., Heidner, E. J., Ladner, J. E., Bettlestone, J. G., Ho, C., & Slade, E. F. (1974) *Biochemistry 13*, 2187-2200.
Perutz, M. F., Sanders, J. K. M., Chenery, D. H., Noble, R. W., Pennelly, R. R., Fung, L. W.-M., Ho, C., Giannini, F., Pörschke, D., & Winkler, H. (1978) *Biochemistry 17*, 3640-3652.
Philo, J. S., & Dreyer, U. (1985) *Biochemistry 24*, 2985-2992.
Savicki, J. P., Lang, G., & Ikeda-Saito, M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5417-5419.
Scholler, D. M., & Hoffman, B. M. (1979) *J. Am. Chem. Soc. 101*, 1655-1662.
Scholler, D. M., Wang, N.-Y. R., & Hoffman, B. M. (1978) *Methods Enzymol. 52*, 487-493.
Verloop, A., Hoogenstraaten, W., & Tipker, J. (1976) in *Drug Design* (Ariëns, E. J., Ed.) Vol. 7, pp 165-207, Academic Press, New York.
Winter, M. R. C., Johnson, C. E., Lang, G., & Williams, R. J. P. (1972) *Biochim. Biophys. Acta 263*, 515-534.
Wüthrich, K. (1970) in *Structure and Bonding* (Hemmerich, R., Jorgensen, C. K., Neilands, J. B., Nyholm, S. R. S., Reinen, D., Williams, R. J. P., Eds.) Vol. 8, pp 53-121, Springer-Verlag, West Berlin.
Yonetani, T., Iizuka, T., Asakura, T., Otsuka, J., & Kotani, M. (1972) *J. Biol. Chem. 247*, 863-868.

Cooperative and Noncooperative Binding of Protein Ligands to Nucleic Acid Lattices: Experimental Approaches to the Determination of Thermodynamic Parameters†

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ABSTRACT: Many biologically important proteins bind nonspecifically, and often cooperatively, to single- or double-stranded nucleic acid lattices in discharging their physiological functions. This binding can generally be described in thermodynamic terms by three parameters: n, the binding site size; K, the intrinsic binding constant; ω, the binding cooperativity parameter. The experimental determination of these parameters often appears to be straightforward but can be fraught with conceptual and methodological difficulties that may not be readily apparent. In this paper we describe and analyze a number of approaches that can be used to measure these protein–nucleic acid interaction parameters and illustrate these methods with experiments on the binding of T4-coded gene 32 (single-stranded DNA binding) protein to various nucleic acid lattices. We consider the following procedures: (i) the titration of a fixed amount of lattice (nucleic acid) with added ligand (protein); (ii) the titration of a fixed amount of ligand with added lattice; (iii) the determination of ligand binding affinities at very low levels of lattice saturation; (iv) the analysis of ligand cluster size distribution on the lattice; (v) the analysis of ligand binding to lattices of finite length. The applicability and limitations of each approach are considered and discussed, and potential pitfalls are explicitly pointed out.

The nonspecific binding of proteins to single- or double-stranded nucleic acid lattices is a central feature of many functional and regulatory biological processes. Theoretically, the problem can be viewed as the binding of large ligands to a one-dimensional lattice, where each ligand interacts with more than one lattice unit (nucleotide residue or base pair), and thus also covers more than one potential ligand binding site (i.e., ligand binding is of the "overlap" type). Overlap binding of ligands to a one-dimensional lattice complicates the analysis of titration curves because the binding sites on the lattice are not titrated independently. Because of overlap, the number of free lattice binding sites occluded per binding event *decreases* with increasing saturation of the lattice. As a consequence, overlap binding is effectively "negatively cooperative", and it becomes progressively more unfavorable to bind additional ligands as lattice saturation is approached. In addition, protein binding to nucleic acid lattices may also be (and generally is) positively cooperative, in that the binding

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