tures were the 6-arm is also folded back toward the core.

A knowledge of the three-dimensional structure of the carbohydrate chains of Asn-linked glycopeptides has brought to light many interesting features of their behavior in solution which in turn has led to a better appreciation of the molecular basis of the specificity in their biosynthesis (Brisson & Carver, 1983c).

Acknowledgments

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Registry No. GGN, 85995-13-7; GGN(GN), 86012-73-9; D3, 86012-74-0; A3, 86012-75-1; Manα1–3Manα1–6Manβ1–4GlcNAcβ, 85995-14-8; Manα1–6(Manα1–3)Manα1–OMe, 68601-74-1.

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Quaternary Structure and Spin-State Transition in Azide Methemoglobin A†

Saburo Neya,* Sakae Hada, and Noriaki Funasaki

ABSTRACT: The temperature-dependent ultraviolet and visible absorption changes of human azide methemoglobin with and without inositol hexaphosphate (IHP) were examined in a 4–35 °C range. The 537-nm absorption change of IHP-free hemoglobin was about 1.2-fold larger than that of IHP-bound hemoglobin. The data were analyzed by considering the thermal spin equilibrium within the R and T conformers and the quaternary equilibrium between the two conformers. The spin equilibrium analysis suggested that the T conformer has a larger high-spin content than the R conformer. The quaternary equilibrium analysis, on the other hand, showed that the T conformer is more populated at lower temperature. The thermodynamic values for the quaternary equilibrium were determined to be $\Delta H = -13.3$ kcal/mol and $\Delta S = -47.6$ eu. The large negative ΔH and ΔS values were compensated for each other to give a small energy difference between the two quaternary states, e.g., $\Delta G_4 = 670$ cal/mol of tetramer at 20 °C. The coincidence of the temperature-dependent IHP-induced changes in the visible and ultraviolet absorptions of heme and aromatic chromophores at the subunit boundaries suggested that the quaternary transition energy is not localized at heme moiety. The reverse temperature dependence of the T conformer fraction as compared with the high-spin fraction of heme iron was interpreted as indicating that the appearance of the T state is not directly coupled with an increase in the strain of Fe–N(F8 His) linkage in azide methemoglobin A.

The increased ligand affinity of oxyhemoglobin relative to deoxyhemoglobin lies in a structural difference between the relaxed (R) and tense (T) quaternary states. The X-ray analyses on the liganded (Ladner et al., 1977) and unliganded (Fermi, 1975) hemoglobins suggested that the iron displacement from the heme plane, linked to the spin state of heme iron, could act as a switch in the quaternary structure (Perutz, 1970). The quaternary structure and spin-state relationship were applied to methemoglobin complexes (Perutz et al., 1974a,b, 1978). On the basis of ligand binding, spectroscopic, and X-ray results, it was found that an allosteric effector IHP[1] is capable of converting high-spin methemoglobin from the R to T states (Fermi & Perutz, 1977; Perutz, 1979). The low-spin methemoglobin exhibited much weaker IHP-induced responses, which were not regarded as to be associated with the quaternary transition. It was, however, found that IHP can promote a substantial conversion of low-spin methemoglobin to the T state (Neya & Morishima, 1981a). The presence of the T state in low-spin methemoglobin seems to be inconsistent with the simple coupling between the position of heme iron and the globin quaternary structure.

To provide an insight into the influence of iron spin state on the globin quaternary structure and to examine the dynamic

† From the Department of Physical Chemistry, Kyoto College of Pharmacy, Yamashina, Kyoto 607, Japan. *Received January 26, 1983.* This work was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

[1] Abbreviations: IHP, inositol hexaphosphate; Pipes, 1,4-piperazinediethanesulfonic acid.

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property of the quaternary equilibrium, we examined azide methemoglobin A in the presence of IHP. The coexistence of the R and T conformers makes azide methemoglobin an ideal system to analyze the quaternary equilibrium and the spin equilibrium simultaneously. We measured the temperature-dependent visible and ultraviolet absorptions of azide methemoglobin A in the presence and absence of IHP. From the analysis of the absorption data, the allosteric constant $L_4 = [T_4]/[R_4]$ was evaluated. The T conformer property, obtained from the present analysis, was compared with those of the azide complexes of carp and human abnormal hemoglobins (Perutz et al., 1978). The thermodynamic parameters associated with the quaternary equilibrium were correlated with possible structural changes in quaternary transition of azide methemoglobin A.

Materials and Methods

Chemicals. Sodium azide was obtained from Nakarai Chemicals, Kyoto, and Pipes was from Dojin Laboratories, Kumamoto. Horse heart myoglobin (type III) and IHP (type V) were purchased from Sigma. All other chemicals were analytical grade and were used without purification.

Hemoglobin. Human blood was drawn from one of the authors (S.N.). Purified methemoglobin was prepared as described previously (Neya & Morishima, 1981a). Azide methemoglobin in 0.1 M NaN_3 plus 0.05 M Pipes, pH 6.43, was left standing for 5 h or longer to complete azide saturation. Heme concentration of azide methemoglobin solution was determined with $\epsilon_{542} = 11.4$ mM^{-1} cm^{-1} at 20 °C (Perutz et al., 1974b).

pH Value. The pH value of hemoglobin solution was measured with a pH meter, Hitachi-Horiba Model M-7II, equipped with a combination electrode, Toko Kagaku Model CE-105.

Absorption Spectra. Visible and ultraviolet absorption spectra were recorded on a Shimadzu MPS-2000 spectrophotometer equipped with a thermostated sample holder. Sample temperature was controlled to ±0.1 °C with a circulating water bath and monitored with a thermistor dipped into the sample cuvette.

Difference spectra were recorded with a pair of matched cuvettes with 1.0-cm path length. Thermal difference spectra were obtained by subtracting the spectrum stored in a computer memory at a starting temperature from the observed curve.

NMR Spectra. Proton NMR spectra at 300 MHz were recorded on a Nicolet NT-300 spectrometer with a temperature variation unit according to the previously reported method (Neya & Morishima, 1981a). The sample volume was 0.3 mL.

Results

IHP-Induced Visible Difference Spectra. In the initial experiment to demonstrate the temperature effect, the IHP-induced visible difference spectra of azide methemoglobin were recorded at the three different temperatures (Figure 1). The IHP addition at 21.4 °C led to the appearance of the peaks at 460, 503, 600, and 632 nm and troughs at 538 and 571 nm. The spectrum is closely similar to those reported for human (Perutz et al., 1974b) and carp (Perutz et al., 1978) azide methemoglobins. It is to be noted that the IHP-induced spectral change is larger at lower temperatures. The absorption difference $\Delta A_{632-571nm}$ increased by 1.16-fold at 3.7 °C and decreased by 0.71-fold at 34.9 °C, as compared with the value at 21.4 °C. Thus the IHP-induced spectral changes in azide methemoglobin are temperature dependent.

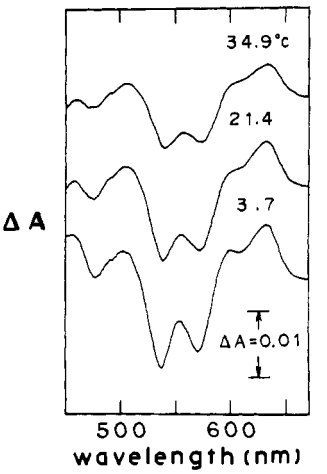


FIGURE 1: IHP-induced visible difference spectra of azide methemoglobin A in 0.1 M NaN_3 plus 0.05 M Pipes, pH 6.43. Heme concentration was 100 μM, and IHP concentration was 8.2 mM.

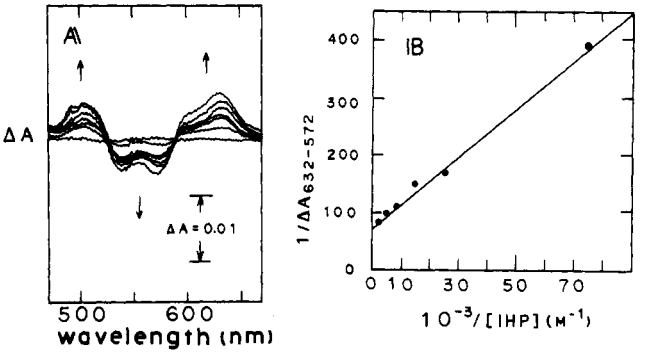


FIGURE 2: IHP binding to stripped azide methemoglobin at 35.6 °C. (A) Visible absorption changes upon addition of IHP in 0.1 M NaN_3 plus 0.05 M Pipes, pH 6.43. IHP concentration increased as indicated by the arrows. (B) Relation between absorption change and IHP concentration. An IHP binding constant (1.88 ± 0.33) × 10^4 M^{-1} was obtained.

It could be questioned, however, whether the smaller spectral changes at higher temperature may be an artifact caused by incomplete IHP binding. The IHP binding constant at 35 °C was determined to check a possible effect of the IHP dissociation. Figure 2 shows the visible difference spectral changes observed in IHP titration. From the analysis of the absorption changes, an IHP binding constant of 1.88 × 10^4 M^{-1} was obtained. This value is about 24-fold smaller than 4.5 × 10^5 M^{-1} determined at 25 °C by Perutz et al. (1974a) for azide methemoglobin. But the binding constant at 35 °C ensures the IHP saturation under the condition described in Figure 1, suggesting that the smaller IHP-induced responses at higher temperatures are not due to the lack of IHP binding but due to a structural change in IHP-bound azide methemoglobin.

Temperature Dependence of the Visible Absorption. For the interpretation of the IHP-induced difference spectra in Figure 1, temperature dependence of the visible absorption in azide methemoglobin was examined in the presence and absence of IHP. Figure 3 shows the thermal difference spectra of stripped azide methemoglobin.[2] With rising temperature, the absorption increased at 460, 499, 597, and 626 nm and decreased at 537 and 571 nm, with isosbestic points at 514

[2] From the absorption changes of stripped azide methemoglobin in Figure 3, an experimental equation for the temperature dependence of the extinction coefficient, $\epsilon_{542} = -0.000038t^2 - 0.0168t + 11.752$ mM^{-1} cm^{-1} ($t = 0 \sim 40$ °C), was obtained, on the basis of $\epsilon_{542} = 11.4$ mM^{-1} cm^{-1} at 20 °C (Perutz et al., 1974b).

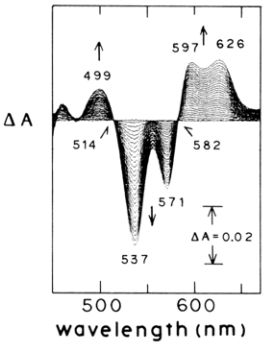


FIGURE 3: Thermal difference spectra of stripped azide methemoglobin in 0.1 M NaN_3 plus 0.05 M Pipes, pH 6.43. Spectra were recorded in a 4.7–34.2 °C range at 1.1 °C intervals. Base line was recorded at 4.7 °C. Temperature increased as indicated by the arrows. Heme concentration was 78.8 μM.

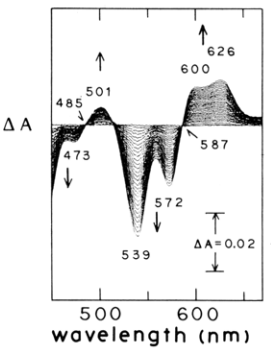


FIGURE 4: Thermal difference spectra of IHP-bound azide methemoglobin in 0.1 M NaN_3 plus 0.05 M Pipes plus 16.1 mM IHP, pH 6.43. Spectra were recorded in a 4.1–34.6 °C range at 1.1 °C intervals. Heme concentration was 78.8 μM.

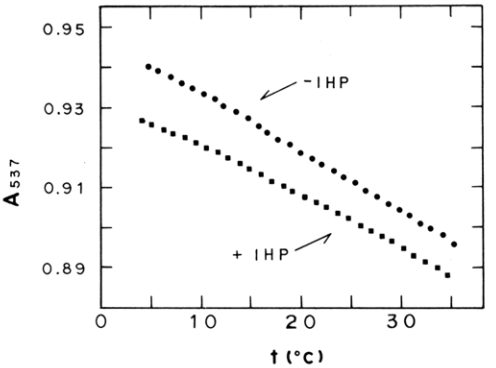


FIGURE 5: Temperature dependence of the 537-nm absorptions of stripped (●) and IHP-bound (■) azide methemoglobins. The data in Figures 3 and 4 were plotted.

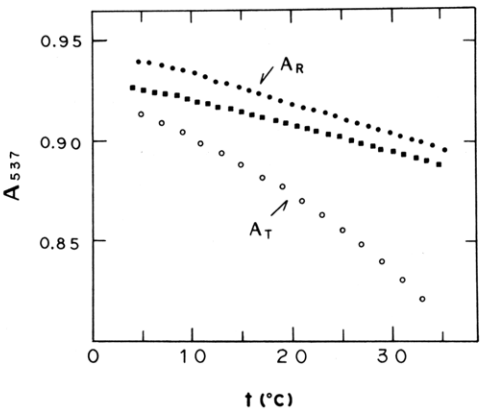


FIGURE 6: Estimated absorbances of the T conformer of azide methemoglobin (○). The observed values in Figure 5 were plotted for comparison.

and 582 nm. The increase in the high-spin bands and the decrease in the low-spin bands at higher temperature are in agreement with the reported results (Cho & Hopfield, 1979) and suggest a shift of the thermal spin equilibrium toward the high-spin state.

The thermal difference spectra of IHP-bound azide methemoglobin, recorded over a similar temperature range, were distinct from those recorded in the absence of IHP. Peaks were observed at 501, 600, and 626 nm, and troughs were at 473, 539, and 572 nm (Figure 4). The changes around 515 nm were not isosbestic. Figure 4 further shows that the magnitude of the spectral changes is smaller as compared with the results in Figure 3. The absorption maxima and minima of the azide methemoglobin spectrum in the presence of IHP are slightly shifted to the red when compared with the spectra recorded in the absence of IHP. This may be interpreted to indicate the mixing of an IHP-induced new conformer and is consistent with the lack of the isosbestic point around 515 nm in Figure 4.

The temperature dependence of the 537-nm absorption, obtained from the results in Figures 3 and 4, is illustrated in Figure 5. The absorption changes of stripped hemoglobin are about 1.2-fold larger than those of IHP-bound hemoglobin. Figure 5 shows that the absorption difference of the hemoglobins with and without IHP increases at lower temperature, consistent with the visible spectral observation in Figure 1.

Estimation of the T Conformer Absorption. The visible absorption of the R conformer is directly monitored for stripped azide methemoglobin. On the other hand, direct observation of the T conformer absorption is not possible because IHP-bound azide methemoglobin is a quaternary equilibrium mixture of the R and T conformers (Neya & Morishima, 1981a). For the estimation of the T conformer absorbance, the temperature-dependent visible absorption changes in Figure 5 are analyzed below.

The absorbance of IHP-bound azide methemoglobin is represented as a weighted average:

$$A = (1 - \alpha)A_R + \alpha A_T$$

where A_R and A_T are absorbances of the R and T conformers, respectively, and α is the T conformer fraction. The A_R values may be experimentally determined for the stripped hemoglobin, as shown in Figure 3.

The A_T value may be evaluated as follows. The ΔH and ΔS associated with the quaternary equilibrium of the fully ligated hemoglobin are calculated from the thermodynamic relation $\Delta G_4 = -RT \ln [T_4]/[R_4] = \Delta H - T\Delta S$, where R is the gas constant and T is absolute temperature. With the above two relations, we obtain a van't Hoff equation:

$$\ln [T_4]/[R_4] = \ln \frac{A_R - A}{A - A_T} = -\frac{\Delta H}{R}\frac{1}{T} + \frac{\Delta S}{R}$$

The "best" values of A_T are defined as those which give linear $\ln [T_4]/[R_4]$ vs. $1/T$ plots for the van't Hoff equation. The estimated A_T's are given in Figure 6. In Figure 6, the A_T and A_R are temperature dependent, reflecting the thermal spin equilibria within the T and R conformers. Quaternary equilibrium, on the other hand, is reflected as the progressive shift of the observed absorbance A toward A_R and A_T at higher and lower temperatures, respectively.

Analysis of the Absorption Changes. IHP-bound azide methemoglobin is a complex system since it contains the R and T conformers which are in spin equilibrium between the high- and low-spin states. The overlapping spin state and

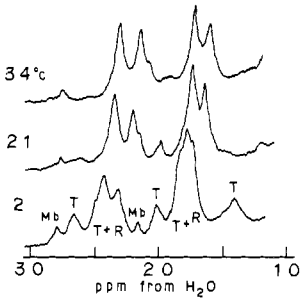


FIGURE 7: Proton NMR spectra of azide methemoglobin in H_2O, pH 6.42, containing 0.1 M NaN_3 plus 0.05 M Pipes plus 0.1 M NaCl plus 10 mM IHP. The spectral intensity was normalized against the ~28-ppm peak of horse metmyoglobin added as an intensity reference. Peaks from the R and T conformers are labeled with R and T. Hemoglobin concentration was 2.6 mM in heme.

quaternary equilibria were separately evaluated with the results in Figure 6.

(A) Spin Equilibrium Analysis. The spin equilibrium between the high- and low-spin states is characterized with an equilibrium constant K = [low spin]/[high spin]. The absorption changes within the R and T conformers in Figure 6 were analyzed on the basis of the spin equilibrium according to the reported procedures (Lange et al., 1980; Makino et al., 1980; Malcolm, 1973). Plots of log K vs. $1/T$ gave straight lines with $\Delta H = -5.81 \pm 0.01$ kcal/mol and $\Delta S = -15.0 \pm 0.1$ eu for the R conformer and $\Delta H = -6.52 \pm 0.01$ kcal/mol and $\Delta S = -20.3 \pm 0.1$ eu for the T conformer. The correlation coefficients (Mortimer, 1981) of the linear plots were r = 0.9972 and 0.9958 for the R and T conformers, respectively.

(B) Quaternary Equilibrium Analysis. Temperature-dependent quaternary equilibrium shift is reflected as the transition of A values in Figure 6 from A_R to A_T with decreasing temperature. The absorption changes were analyzed with the above van't Hoff equation. With the limiting A_T and A_R values at each temperature, the van't Hoff plots for the quaternary equilibrium were obtained with r = 0.9985, corresponding to $\Delta H = -13.3 \pm 0.1$ kcal/mol and $\Delta S = -47.6 \pm 0.3$ eu. The results suggest that the quaternary equilibrium is shifted to the T state with decreasing temperature. The temperature where the R and T conformers are equally populated was determined to be 279.0 ± 3.6 K with these ΔH and ΔS.

Proton NMR Spectra. Proton NMR spectra of IHP-bound azide methemoglobin were recorded to evaluate the L_4 value estimated from the visible absorption analysis. Figure 7 shows the intensity-calibrated NMR spectra of IHP-bound azide methemoglobin A. Peaks at ~26, ~20, and ~14 ppm have been assigned to the T conformer (Neya & Morishima, 1980a, 1981a). The chemical shifts of the R and T conformer peaks are temperature dependent reflecting the spin equilibria within each of the conformers (Neya & Morishima, 1980a; Morishima et al., 1978). The ~26-ppm peak, which has been used as a T marker, is dominant at 2 °C while it is scarcely observed at 34 °C. The intensity of this peak is about 3-fold larger at 2 °C than at 21 °C. With rising temperature from 21 to 34 °C, the intensity decreased by about 3-fold. The changes in the peak intensity are in agreement with those estimated from the thermodynamic values obtained by the above quaternary equilibrium analysis.

Ultraviolet Difference Spectra. The ultraviolet difference spectrum has been used to monitor quaternary structural change in hemoglobin (Perutz et al., 1974a, 1978). The IHP-induced ultraviolet difference spectra of azide methemoglobin were recorded to examine the temperature effect on the subunit boundaries. Figure 8 shows that the 270- and

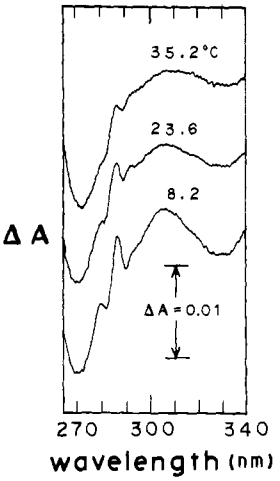


FIGURE 8: IHP-induced ultraviolet difference spectra of azide methemoglobin in 0.1 M NaN_3 plus 0.05 M Pipes at pH 6.43. Heme concentration was 36 μM, and IHP concentration was 12 mM.

287-nm peaks from Tyr-α42 and the 294- and 303-nm peaks from Trp-β37 (Perutz et al., 1974a) are about 4-fold larger at 8.2 °C than at 35.2 °C. Thus the IHP-induced peaks are intensified with lowering temperature, demonstrating that temperature can perturb the subunit boundary structure. This result is comparable with the heme electronic absorption (Figure 1) and proton NMR spectra (Figure 7), which reflect structural changes at the direct heme vicinity.

Discussion

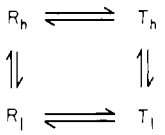
Temperature-Dependent Quaternary Equilibrium Shift. Present results show that the IHP-induced visible and ultraviolet absorptions of azide methemoglobin are intensified at lower temperature. These results are comparable with the previous NMR results on 1-vinylimidazole-ligated methemoglobin to show that the intensity of the T marker increases at lower temperature (Neya & Morishima, 1981a). The thermodynamic values obtained for azide methemoglobin allow us to calculate at any temperature the allosteric constant $L_4 = [T_4]/[R_4]$. At 50 °C, L_4 = 0.04, and azide methemoglobin in the presence of IHP is primarily in the R state. At 0 °C, L_4 = 1.67, and the T conformer is predominantly populated. The L_4 value increases by about 40-fold in going from 50 to 0 °C.

The energy difference between the two quaternary states of IHP-bound azide methemoglobin is $\Delta G_4 = -RT \ln L_4$ = 670 cal/mol of tetramer at 20 °C. The small energy difference between the two states is due to the compensation of ΔH with ΔS. The large negative ΔH and ΔS values of the quaternary equilibrium are consistent with the formation of several bonds and the decrease in the freedom of globin on the R to T transition and suggest that the quaternary transition energy is not localized in a specific linkage. This idea may be supported by the observations in Figures 1 and 8, which show that the alteration at the heme vicinity coincides with the changes in the subunit boundaries. Hopfield (1973) suggested that the quaternary transition energy is not localized at heme but stored as small strain over hemoglobin molecule. The present result seems to be consistent with his idea.

Increase in the quaternary equilibrium constant at lower temperature has been reported also for fish and worm hemoglobins. From the flash photolysis study of CO binding, Brunori et al. (1980) found that the L value of trout deoxyhemoglobin changes from 6.4 at 72 °C to 3×10^3 at 20 °C. Saffran & Gibson (1979) and Morris & Gibson (1982) reported similar results for menhaden and tuna hemoglobins,

respectively. Wittenberg et al. (1981) showed that the L value of a tube worm deoxyhemoglobin increases at lower temperature. The temperature effect on IHP-bound azide methemoglobin A is in the same direction with the fish and worm hemoglobins. This may suggest that a shift of the quaternary equilibrium to the T state at lower temperature is not specific to the hemoglobins of lower animals but a general phenomenon of hemoglobins.

Determination of the Microscopic Allosteric Constants. It is interesting to determine the allosteric constant [T]/[R] for the purely high- and low-spin species of IHP-bound azide methemoglobin. The following scheme was considered:



where h and l refer to the high- and low-spin states, respectively. The [l]/[h] ratio may be determined from the ΔH and ΔS values of the thermal spin equilibria of the R and T conformers. The ratios are $[R_l]/[R_h] = 11.6$ and $[T_l]/[T_h] = 2.5$ at 20 °C. The macroscopic quaternary equilibrium constant, calculated from the thermodynamic parameters of the quaternary equilibrium analysis, is $L_4 = ([T_l] + [T_h])/([R_l] + [R_h]) = 0.32$ at 20 °C. With these three relations, the ratio of each conformer is evaluated to be $[R_l]:[R_h]:[T_l]:[T_h] = 0.70:0.06:0.17:0.07$. The microscopic allosteric constants for the purely high- and low-spin species are $L_h = [T_h]/[R_h] = 1.2$ and $L_l = [T_l]/[R_l] = 0.25$. The L_h value of 1.2 suggests that the purely high-spin species cannot be completely switched into the T state and that it is a quaternary equilibrium mixture. The same is true of the low-spin species with $L_l = 0.25$.

Properties of the T Conformer. We compared the T conformer property of azide methemoglobin A with that of other azide methemoglobins in the T state. As calculated above, the [l]/[h] ratios are 11.6 for the R conformer and 2.5 for the T conformer at 20 °C. An increased high-spin fraction in the T conformer is comparable with the results for carp and human abnormal hemoglobins. The [l]/[h] ratios are reported to be 9.1 for the R conformer and 1.2 for the T conformer of carp azide methemoglobin (Perutz et al., 1978). Similar results were reported for the azide complex of hemoglobin M Milwaukee, for which the [l]/[h] ratios are ≥15 and 3.7 for the R and T conformers, respectively (Perutz et al., 1978). Thus, the characteristic of the T conformer of azide methemoglobin A is similar to that of the azide complexes of carp and human abnormal hemoglobins.

Coupling between Quaternary Structure and Spin State. The stereochemistry of heme is an essential factor to regulate the ligand affinity of hemoglobin. The presence of heme tension in the T state has been demonstrated with various methods (Perutz et al., 1978; Maxwell & Caughey, 1976; Messana et al., 1978; Nagai & Kitagawa, 1980; Neya & Morishima, 1981b). Though the coupling between the quaternary structure and the spin state has been assumed (Perutz, 1979), the influence of the iron spin state on the globin quaternary structure has not been examined directly. IHP-bound azide methemoglobin provides a unique examination of the coupling between the quaternary structure and the spin state because the spin equilibrium and the quaternary equilibrium simultaneously change with temperature.

Present results show that the spin equilibria of the T and R conformers are shifted to the high-spin side with increasing temperature. The increased high-spin fraction may be structurally related to an increased strain in the Fe–N(His F8) bond, in view of the visible absorption and magnetic studies of the mixed-spin derivatives of methemoglobin (Perutz et al., 1978; Messana et al., 1978) and the model complex study of thermal spin equilibrium of azide hemoprotein (Huang & Kassner, 1979; Neya & Morishima, 1980b, 1982).

However, it is to be noted that the T conformer population of azide methemoglobin increases with decreasing its high-spin content. The T conformer fractions, calculated from the present results, are 0.04 at 50 °C and 0.63 at 0 °C, while the high-spin fractions of the T conformer are estimated to be 0.42 at 50 °C and 0.04 at 0 °C from the spin equilibrium analysis. This result may be interpreted as indicating that, though the globin quaternary structure affects the iron spin state of azide methemoglobin A, the low- to high-spin change of iron does not directly facilitate the transition of globin to the T state. Thus, simple coupling between the quaternary structure and the spin state seems unlikely.

It has been reported for methemoglobin A bound with IHP that $L_4 \gg 1$ in the high-spin complex (Perutz et al., 1974b) and that $L_4 \approx 1$ in the low-spin complex (Neya & Morishima, 1981b). However, in view of the direct examination of the influence of the iron spin state on the quaternary structure in azide methemoglobin A, the apparent dependence of L_4 of methemoglobin A on the iron spin state is not directly correlated to the changes in the iron displacement from the heme plane. This idea is consistent with the presence of the T state in the low-spin complexes of ferrous (Perutz et al., 1976) and ferric (Neya & Morishima, 1981a) hemoglobin A and suggests the structural changes which do not localize at heme proximal site to initiate the quaternary transition of hemoglobin.

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Registry No. Azidomethemoglobin A, 9072-23-5; IHP, 83-86-3.

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Stoichiometry of Manganese and Calcium Ion Binding to Concanavalin A†

C. Fred Brewer,* Rodney D. Brown, III, and Seymour H. Koenig

ABSTRACT: Using measurements of solvent nuclear (proton) magnetic relaxation dispersion (NMRD), we have previously shown that concanavalin A (Con A) can exist in two conformational forms and that, in the absence of Ca^{2+}, Mn^{2+} can bind to both the S1 and S2 sites of each monomer of Con A of at least one conformer [Brown, R. D., III, Brewer, C. F., & Koenig, S. H. (1977) *Biochemistry 16*, 3883–3896]. Recently other investigators have claimed that the stoichiometry of Mn^{2+} binding to Con A is only 1:1 for this conformational state, both in the absence and presence of saccharide; the same was claimed for Ca^{2+} under similar conditions. We now present titration and equilibrium dialysis experiments, both in the absence and presence of saccharide, using NMRD and atomic absorption spectroscopy, to investigate the stoichiometry of Mn^{2+} and Ca^{2+} binding to Con A. We have extended the NMRD method to include the determination of the total concentration of Mn^{2+} in samples of Con A. This, coupled with our previous use of NMRD to measure the concentration of free Mn^{2+} in protein solutions as well as the distribution of bound Mn^{2+} among different sites, allows us to measure the stoichiometry of binding with precision. We reconfirm that, at equilibrium in the presence of excess Mn^{2+}, the binding stoichiometry of Mn^{2+} to Con A is 2:1, both in the absence and presence of saccharide. Addition of Ca^{2+} to a solution of Mn^{2+}–Con A results in stoichiometric displacement of Mn^{2+} from the S2 site under the conditions investigated. Under nonequilibrium conditions, Mn^{2+} forms a metastable binary complex with the protein that persists for days at 5 °C. We also report, for the first time, values for all of the dissociation constants of binary and ternary complexes of Mn^{2+} with both conformations of Con A in solution. Atomic absorption measurements also indicate that Ca^{2+}, in the absence of Mn^{2+}, binds to both S1 and S2 sites in the absence and presence of saccharides.

Concanavalin A (Con A),[1] a metalloprotein isolated from the jack bean (*Canavalia ensiformis*), has attracted considerable interest as a probe for investigating the properties of cell surfaces (cf. Bittiger & Schnebli, 1976). Its utility is related to its saccharide binding activity, which is known to be influenced by the binding of metal ions. Each monomeric unit of Con A can bind divalent metal ions at two sites: S1, the "transition-metal" site, and S2, the "calcium" site. S2 is formed once S1 is occupied (Kalb & Levitzki, 1968). Considerable attention has been focused on the details of metal ion interactions with these two sites, interactions between the two sites, and the manner in which saccharide binding activity is controlled by their occupancy.

Brown et al. (1977) and Koenig et al. (1978), using solvent proton nuclear magnetic relaxation dispersion measurements (NMRD) to study the interaction of Mn^{2+} and Ca^{2+} with apo-Con A, concluded that binding of Mn^{2+} to S1 and Ca^{2+} to S2 forms a metastable state with a predominantly inactive conformation, called "unlocked", which converts to an active conformation, called "locked". The ground state free energies of the two conformations differ by only a few kcal M^{-1}, with the sign of this difference determined by the occupancy of S1 and S2 by metal ions. A relatively high energy barrier of about 22 kcal M^{-1}, which has been associated with a cis–trans isomerization in the polypeptide backbone of the protein (Brown et al., 1977), separates the two conformations, making their interconversion relatively slow. A major distinction in the properties of the two conformations is that the locked form has a much greater affinity for metal ions and for saccharides. These conclusions are consistent with earlier stopped-flow NMR studies of Con A reported by Grimaldi & Sykes (1975) and have been confirmed by subsequent polarographic studies of the kinetics of binding of saccharide and a variety of metal ions to Con A by Sherry et al. (1978), fluorescence stopped-

† From the Departments of Molecular Pharmacology, and Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York 10461 (C.F.B.), and the IBM Thomas J. Watson Research Center, Yorktown Heights, New York 10598 (S.H.K. and R.D.B.). *Received November 30, 1982.* This work was supported in part by Grant CA-16054, awarded to C.F.B. by the National Cancer Institute, Department of Health, Education, and Welfare, and Core Grant P30 CA-13330 from the same agency.

[1] Abbreviations: Con A, concanavalin A; NMRD, nuclear magnetic relaxation dispersion; α-MDG, methyl α-D-glucopyranoside; MUM, 4-methylumbelliferyl α-D-mannopyranoside.

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