Fluctuation of the Myoglobin Structure

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The kinetics of hydrogen-deuterium exchange in sperm whale metmyoglobin has been followed in aqueous solutions of various pD values (5.6—7.7) and at various temperatures (0—55 °C) by an infrared absorption measurement. At pD=5.6 and at 0 °C, 117 out of the total 148 peptide hydrogen atoms exchange relatively slowly with a rate of an apparent first-order reaction for one hour of the reaction time. This number (117) is almost equal to the sum of the number of peptide NH-groups (108~111) which are found to be involved in hydrogen bonds with the carbonyls of other peptide groups and those (8) which are in hydrogen bonds with oxygen atoms of some sidechains in the myoglobin molecule in the crystalline state. At higher temperatures and/or at higher pD's, however, only 109 peptide NH groups (assignable to those involved in the peptide-peptide hydrogen bonds) are found to exchange relatively slowly. The kinetics of the exchange reactions of these 109 peptide NH groups are explained by assuming that the fluctuation amplitude (*i.e.*, the probability of finding the peptide-peptide hydrogen bonds broken) becomes lower on going from the terminus towards the inner portion in each of the eight α-helices involved in the myoglobin structure.

In our previous works[1-3] it has been shown that a kinetic study of the hydrogen-deuterium exchange reaction of a protein in aqueous solution can provide useful information on the fluctuation of the protein structure. The kinetic data have been connected with the fluctuation of the protein structure through the following two assumptions: (1) The exchange reaction of a peptide NH group is distinctively slow when it is involved in a peptide-peptide hydrogen bonds. (2) The rate constant of such a slow exchange reaction is proportional to the chance of finding the hydrogen bond broken. These assumptions are essentially based upon the reaction scheme proposed by Hvidt[4] many years ago, and by our recent experiments good pieces of evidence have been found for the validity of these assumptions in the case of lysozyme.[1]

In this work myoglobin is the subject of our kinetic measurement. On the hydrogen exchange in myoglobin, Englander and Staley[5] and Abrash[6] made detailed studies. In the former work, special attention was paid to the estimation of the free and hydrogen-bonded peptide NH groups. While, in the latter work the effect of the pH-induced denaturation was intensively examined. Both of the results are interpretable by considering that the assumptions given above are valid. We have extended the hydrogen-exchange measurement into the temperature range of 0 to 55 °C and attempted to discuss a detail of the fluctuation of the structure of this protein in solution.

Experimental

Crystalline sperm whale myoglobin was obtained from Miles-Seravac, Ltd., Maidenhead, Berkshire, England (Batch 57A). The hydrogen-deuterium exchange reaction was followed by infrared absorption measurements according to the method described by Blout, de Lozé and Asadourian.[7] The experimental procedure is the same as given in our previous papers.[1,2,8] The concentration of myoglobin was kept at 3.3%. The solvent was 0.1 M phosphate buffer. The amount of undeuterated peptide group was taken as proportional to the ratio of the absorbance of amide II (at 1550 cm^{-1}) to that of amide I (at 1650 cm^{-1}) bands, A(amide II)/A (amide I). In the absorbance measurements, the baseline for the amide I band was drawn parallel to the 100% transmittance line from the transmittance at 1800 cm^{-1}, and the baseline of the amide II band was taken as the absorption of a solution of the completely deuterated myoglobin. The latter was obtained by keeping the solution of the exchanging protein at pD≒10 and at 45—50 °C for several hours, and then by bringing the pD and temperature of the solution into proper values.

Results and Interpretations

(a) General Features of the Exchange Kinetics. The hydrogen-deuterium exchange reaction was followed for myoglobin in D_2O solutions of pD 5.6, 6.3, 7.0, and 7.7 at 15, 25, 35, 45, and 55 °C. For solution of pD 5.6, the observation was made also at 0 °C and 5 °C. The results are shown in Figs. 1 and 2. As may be seen in these figures, the reaction rate is higher

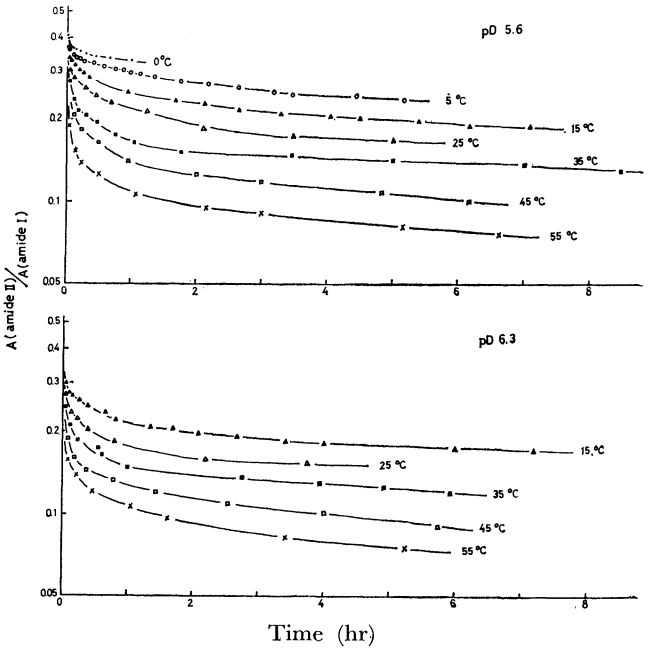

Fig. 1. Semilogarithmic plots of deuterium exchange of myoglobin in D_2O solutions of pD 5.6 (upper) and of pD 6.3 (lower) at various temperatures.

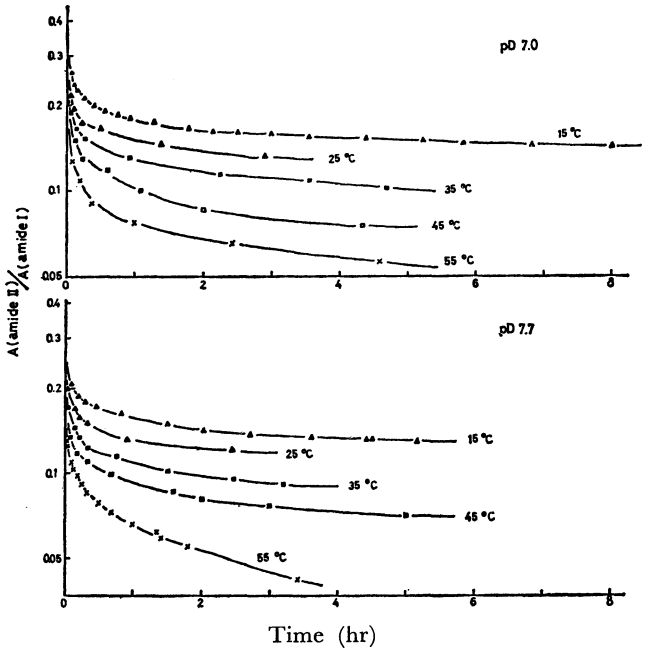

Fig. 2. Semilogarithmic plots of deuterium exchange of myoglobin in D_2O solutions of pD 7.0 (upper) and of pD 7.7 (lower) at various temperatures.

at higher temperatures and in solutions with higher pD's. Under any of these conditions the reaction does not proceed as a simple first-order reaction. This is in contrast to the case of lysozyme,[1,2] where the peptide hydrogen atoms exchange with a rate of an apparent first-order reaction except in an early stage of the reaction. This fact indicates that the peptide NH group in the myoglobin molecule should be classified into a number of categories with different kinetic behaviors from one another.

(b) Measurement of the Free and Hydrogen-bonded Peptide NH Groups. At 0 °C the reaction proceeds so slowly that we can estimate the A(amide II)/A(amide I) value at time zero by an extrapolation (Fig. 3, upper portion). The value is found to be 0.450. This value is equal to what are determined in a less ambiguous for lysozyme[1,2] and α-lactalbumin.[3] The reaction

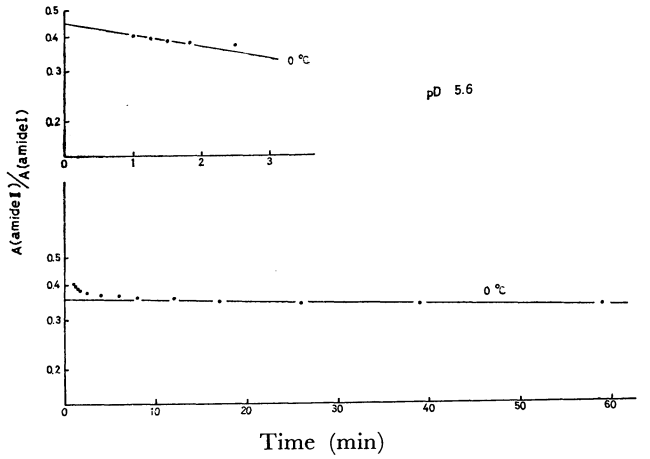

Fig. 3. Semilogarithmic plots of an early stage of the deuterium exchange reaction of myoglobin in a D_2O solution of pD 5.6 at 0 °C.

proceeds rather rapidly in the period of 0 to 10 minutes, and suddenly at about 10 minutes it slows down (see Fig. 3, lower portion). In the period of 17—60 min the exchange proceeds apparently as a simple first-order reaction. The extrapolation of the straight line obtained in the semilogarithmic plots for this stage of the reaction gives an A(amide II)/A(amide I) value of 0.355 at the zero time. This fact indicates that we can divide the peptide NH groups in myoglobin into two main classes; $(0.450-0.355)/0.450=21._0\%$ of them exchange rapidly and the rest $0.355/0.450=79._0\%$ do slowly. Because there are 148 peptide NH groups in the myoglobin molecule, the former corresponds to 31 and the latter 117 in the actual number. According to the crystallographic study of Kendrew *et al.*,[9] 108~111 peptide NH groups in the myoglobin molecule are involved in intramolecular peptide-peptide hydrogen bonds, 8 NH's are involved in intramolecular hydrogen bonds with oxygen atoms in sidechains, and 29—32 are free from such intramolecular hydrogen bonds.[10] Therefore, it is probable that the slowly exchanging 117 peptide NH groups found in our experiment correspond to the $(109+8)\pm2$ peptide NH groups involved in hydrogen bonds in the myoglobin molecule in its crystalline state.

Similar estimation of the free and hydrogen-bonded amides of myoglobin was made by following the tritium exchange by Englander and Staley.[5] Our result is practically equal to theirs. It may be pointed out here, however, that we can extract only the behavior of the peptide NH groups in this protein molecule by the infrared absorption measurement, while in the measurement of ^3H radioactivity one needs to take, in principle, all the exchangeable hydrogen atoms into account.

As may be seen in Fig. 1 (upper), the exchange reaction at 5 °C and at pD=5.6 proceeds as a simple first-order reaction from 18 to 100 minutes. In the semilogarithmic plot, the straight line which corresponds to this part of the reaction intersects the ordinate (A(amide II)/A(amide I)) at 0.330. At 15 °C a straight line in the semilogarithmic plot occurs for the 0~20 minutes period. This also intersects the ordinate at 0.330. This value of A(amide II)/A(amide I) corresponds to 109 peptide NH groups. This is equal to the number of peptides NH groups involved in the peptide-peptide hydrogen bonds. This fact therefore may be interpreted as indicating that, at temperatures higher than 5 °C, only the peptide NH groups involved in the peptide-peptide hydrogen bonds should be classified as the "slowly exchanging peptide NH groups", and those involved in the peptide-sidechain hydrogen bonds are to be classified as the "fast" NH groups. Such an interpretation has been found to be valid for lysozyme[1,2] and for α-chymotrypsin.[11]

(c) Hvidt's "Relaxation Spectra" and the "Fluctuation Amplitude" γ. On the basis of the interpretation just described, we now attempt an analysis of the hydrogen-deuterium exchange reaction with special attention on the 109 peptide NH groups involved in the peptide-peptide hydrogen bonds. For such an analysis, Willumsen[12,13] and Hvidt and Wallevik[14] proposed to display the kinetic data as plots of the number of

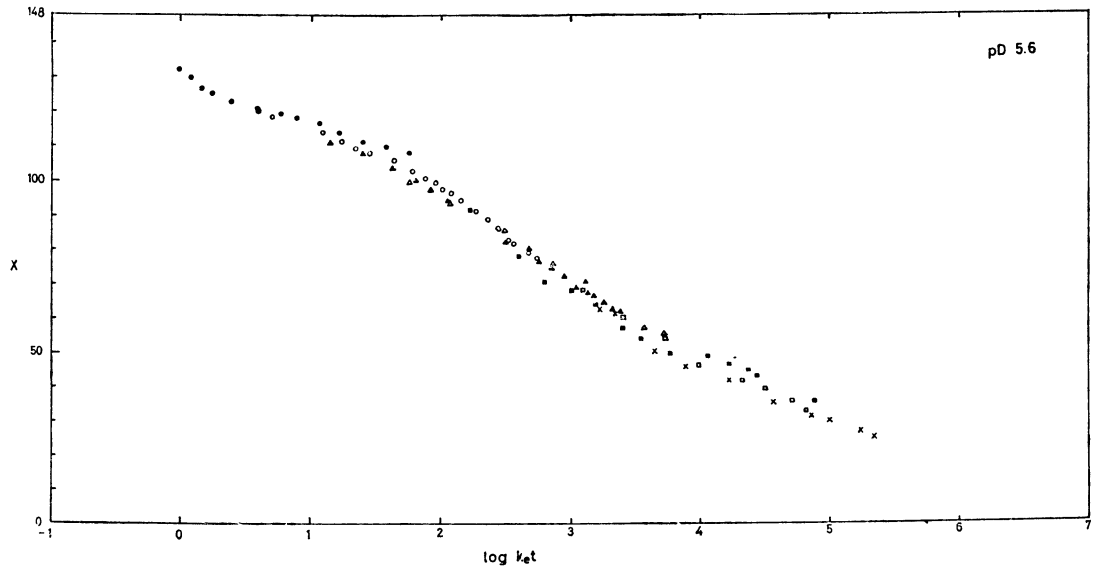

Fig. 4. A re-plotting of the experimental data given in Fig. 1 (upper). X is the number of unexchanged peptide NH groups at time t, and k_e is the rate constant of peptide hydrogen atoms free from hydrogen bonds, calculated according to equation (1). ●, at 0 °C; ○, at 5 °C; ▲, at 15 °C; △, at 25 °C; ■, at 35 °C; □, at 45 °C; and ×, at 55 °C. pD=5.6.

unexchanged peptide hydrogen atoms (X) at a given time (t) *versus* $\log_{10}(k_e t)$, where k_e is the exchange rate constant of solvent-exposed peptide groups. They mentioned such plots as "relaxation spectra". We have re-plotted our kinetic data according this proposal. For k_e at various pD's and temperatures (θ °C), we have adopted, according to Hvidt and Nielsen,[15] the values given by the following formula:

$$k_e = 50(^{0.3-pD} + 10^{pD-6.3})10^{0.05(\theta-20)}\text{min}^{-1} \quad (1)$$

(We have neglected here the primary-structure effects discussed by Molday, Englander and Kallen.[16]) In Fig. 4, an example of the "relaxation spectra" is shown for the peptide NH groups of myoglobin in the solution of pD 5.6; this is obtained by re-plotting the data given in Fig. 1 (upper).

Hvidt and Nielsen[15] showed that most studies of the hydrogen exchange led to the conclusion that the so-called EX_2 reaction mechanism offers the simplest, and the most likely explanation of the slow exchange of the hydrogen-bonded peptide NH groups. Let us consider i-th peptide NH group. The exchange takes place as follows:

$$N_i \underset{k_{2i}}{\overset{k_{1i}}{\rightleftharpoons}} D_i \xrightarrow{k_e} \text{exchange}, \quad (2)$$

where N_i means a state of the i-th NH group involved in a hydrogen-bond and D_i is that after the hydrogen-bond is broken so that it is now ready to exchange with the rate costant k_e. The EX_2 mechanism corresponds to the case of

$$k_e \ll k_2, \quad (3)$$

and in this case X_i (fraction remaining unchanged) is given as

$$X_i = \exp(-\gamma_i k_e t), \quad (4)$$

where

$$\gamma_i = [D_i]/([N_i]+[D_i]) \quad (5)$$

is the probability of finding the particular peptide-peptide hydrogen bond broken so as to be ready to exchange with the rate constant k_e. If the NH group now in question is intrinsically free from hydrogen bond, $\gamma_i \fallingdotseq 1$. While, if it is involved in a peptide-peptide hydrogen bond in the "intact" form of the myoglobin molecule, the γ_i value is very small (in comparison with 1), and such γ_i value may be taken as a measure of the "fluctuation amplitude" of the local structure around the i-th peptide NH group.

Now, according to Eq. (4), the plot of X_i *versus* $\log_{10}(k_e t)$ for a fixed γ_i value should result in a curve whose slope is everywhere independent of the γ_i value, because $dX_i/d\log(k_e t) = 2.30 X_i \ln X_i$. Therefore, the curves corresponding to different γ values have the same shape but are mutually displaced along the abscissa. The amount of displacement of each curve from that with $\gamma_i = 1$ is equal to $-\log_{10}\gamma_i$. The ordinate X in the observed plot such as shown in Fig. 4 should correspond to the sum of X_i at every $\log_{10}(k_e t)$.

$$X = \sum_i \exp(-\gamma_i k_e t) \quad (6)$$

Thus, Hvidt's "relaxation spectrum", *i.e.*, the X *versus* $\log_{10} k_e t$ plot, is considered to be a so-to-say "spectrum" of γ, the probability of finding each NH group free from a hydrogen bond so that it is ready to exchange with the rate constant k_e. In other words, it is a "spectrum" of the amplitude of local fluctuation of the protein structure.

(d) Effects of Temperature and pD *on the Local Fluctuation.* In Fig. 4, observed X *versus* $\log_{10} k_e t$ curves at different temperatures are found to be nearly superposed on one another, although a little more detailed inspection indicates a probably significant tendency that a curve for higher temperature is shifted very slightly towards leftside direction (*i.e.*, towards higher γ value) from a curve for lower temperature. This

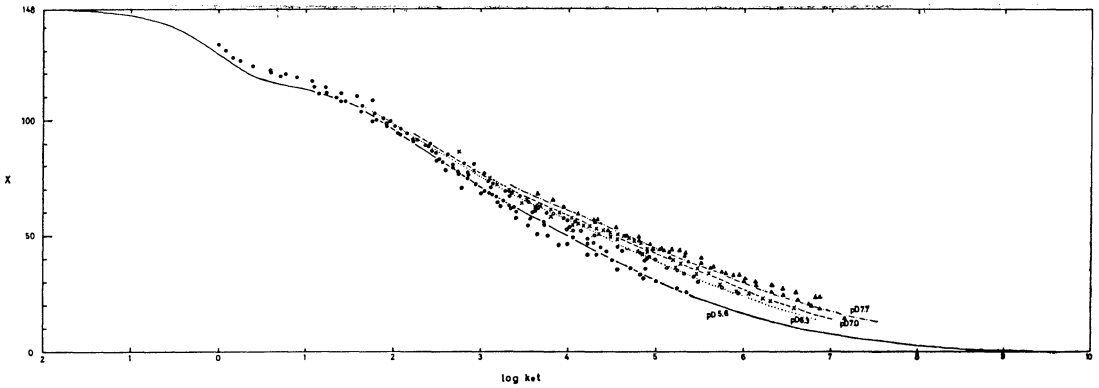

Fig. 5. Observed and calculated "relaxation spectra" of myoglobin. X is the number of unexchanged peptide NH groups at time t. k_e is the rate constant of solvent-exposed peptide hydrogen atoms (free from hydrogen bonds). Points are obtained by re-plotting the experimental data given in Figs. 1 and 2. ●, at pD=5.6 (at various temperatures in the range of 0—55 °C); ○, at pD=6.3; ×, at pD=7.0; ▲, and at pD=7.7. Continuous lines are obtained by calculation on equation (6), and by assigning the γ_i values to the 148 peptide NH groups as shown in Table 1. ——, for pD=5.6; ·····, for pD=6.3; ----, for pD=7.0; and —·—, for pD=7.7.

was found to be the case not only for the plot at pD=5.6 but also for plots at pD=6.3, 7.0, and 7.7. This fact may be taken as indicating that the observed temperature effect on the exchange kinetics is mostly attributable to the temperature effect on k_e, and that γ_i value of every NH group is almost independent of temperature in the range of 0—55 °C, although it becomes very slightly higher on raising the temperature of the solution. This is quite similar to what we found for lysozyme[2] and α-lactalbumin[3] as long as the temperature was kept by 20—25 °C lower than the thermal transition point.

In Fig. 5, we have re-plotted our kinetic data given in Figs. 1 and 2 in the form of X *versus* $\log_{10} k_e t$ curve. On the basis of what has just been stated, no distinction is made here between the points for different temperatures, but different marks are given to points for different pD's. As may be seen in the figure, the "relaxation spectrum" depends slightly but appreciably upon pD. Every γ_i value is considered to become lower as pD of the solution increases from 5.6 to 7.7.

Discussion

The denaturation of sperm whale myoglobin was extensively investigated by Acampora and Hermans[17] as a function of pH and temperature. In Fig. 6, transition temperatures found by them are shown as a function of pH of the solution. The area with hatching corresponds to the native form region. All the place within this region are considered to be similar as far as the optical density and/or optical rotation measurements are concerned. In the manner of fluctuation of the structure, however, there may be a variety even within the native region, and this is just the subject of our study. In this respect, we have examined in the present work the region surrounded by the rectangle in Fig. 6. Within this region, the fluctuation has been found to be greater for lower pH than that for higher pH, while it is almost independent of the temperature of the solution.

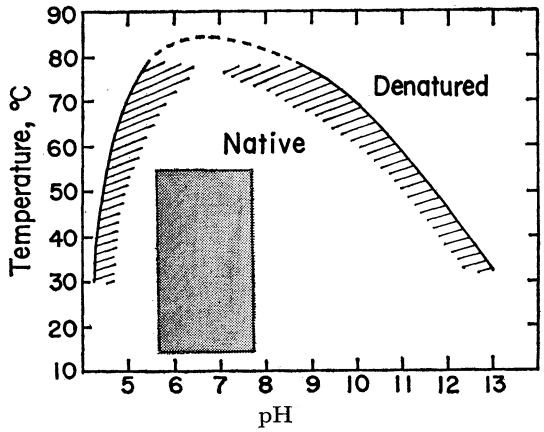

Fig. 6. An equilibrium diagram between the native and denatured forms of myoglobin given by Acampora and Hermans[17]. The rectangular region is what we have examined as to the fluctuation of the structure.

In addition, we have been able to go into some details of the fluctuation of the local structures within the molecule. Some experiments are yet to be designed for determining the rate of hydrogen exchange reaction of every particular NH group, so that the fluctuation amplitude of the structure can be mapped at every exact locus within the molecule. Even by our present study, however, it has been indicated that the map of the local fluctuation is different in myoglobin from that in lysozyme. In the latter, all of the 44 peptide-peptide hydrogen bonds have nearly equal chances of cleavage to one another, while in the former, 109 peptide-peptide hydrogen bonds have various chances, greatly different from one another.

At present, we have no unique solution as for the assignment of each γ_i value to a particular peptide NH group in the molecule. It is not improbable, however, that a peptide NH in a central portion of an α-helix has a lower γ value than that of a peptide NH around the terminal portion of the α-helix. This is just what has often been postulated for explaining various be-

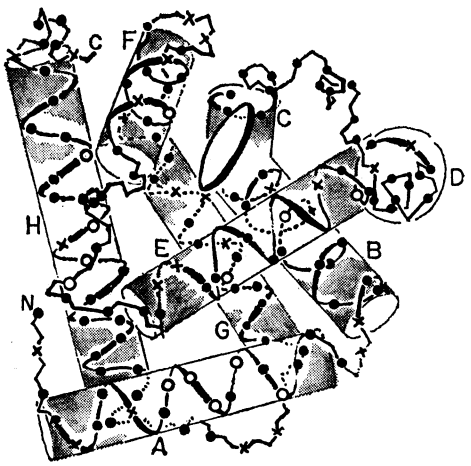

Fig. 7. A schematic drawing of a model of the fluctuation of the myoglobin structure, *i.e.*, a model of the distribution of the γ value in the molecule. γ is the probability of finding each NH···OC hydrogen bond broken, and this is considered to be greater at the terminal portions of each α-helix, and becomes smaller on going towards the central portion. This model is considered to be supported to some extent by a recent neutron diffraction study by Schoenborn.[20] Black and white circles in the figure indicate the deuterated and undeuterated peptide NH groups found by them in a partially deuterated myoglobin crystal. × denotes the absence of any indication for H or D.

haviors of α-helices of synthetic polypeptides.[8,18,19] We found, for example, that the hydrogen-deuterium exchange reaction is slower for longer α-helix of poly-L-glutamic acid.[8] In addition, recent neutron diffraction study of myoglobin by Schoenborn[20] may be taken as supporting the above idea to some extent. By soaking a myoglobin crystal in a solvent with 75% D_2O, they obtained a partially deuterated crystal, and then they located the hydrogen and deuterium atoms in the myoglobin molecule. As may be seen in Fig. 7, the undeuterated peptide groups found by them are mostly located in the central portions of eight α-helices in the molecule.

On the basis of what has just been described, we attempted to construct a tentative model in which the γ value of the peptide NH becomes lower on going from the terminus into the inner portion of each α-helix. The γ value should be proportional to $\exp(-\Delta F_i/kT)$ where ΔF_i is the effective free energy change caused by the N_i—D_i transition, k the Boltzmann constant, and T the absolute temperature. Let us postulate that the free energy difference ΔF_n for the n-th residue from the terminus is given as

$$\Delta F_n = \Delta F_t + (n-1)\Delta F_s. \quad (7)$$

This is based upon the assumptions that the terminus peptide NH group has the free energy change ΔF_t and that the breaking of every peptide-peptide hydrogen bond in an α-helix takes place always after the next-door peptide-peptide hydrogen bond which is nearer to the terminus has been broken, with an additional free energy change of ΔF_s. After a few trials, we found that the following set of the values for the two parameters fits the observed relaxation spectra: $\Delta F_t=2.86$ and $\Delta F_s=0.82$ kcal/mol at pD=5.6 (25 °C), 3.00 and 0.93 at pD=6.3, 3.00 and 0.98 at pD=7.0, and 3.13 and 1.02 at pD=7.7. This set of values of ΔF_t and ΔF_s corresponds to a set of γ values given in Fig. 8 and Table 1. The calculated X *versus* $\log_{10}(k_e t)$

Table 1. A proposed set of γ values which fits the observed relaxation spectra

Character of the peptide NH group	$\log_{10}\gamma$ value, assigned at pD= 5.6	6.3	7.0	7.7	Number of the peptide NH groups in the non helical regions	in helix A	B	C	CD	D	E	F	G	GH	H	Total
Free	0	0	0	0	31											
H-bonded with O atoms in side-chains	−1.5	−1.5	−1.5	−1.5	8											
In the terminal residue in an α-helix	−2.1	−2.2	−2.2	−2.3		2	2	2	2	2	2	2	2	1	2	19
In the 2nd residue	−2.7	−2.9	−2.9	−3.0		2	2	1	1	2	2	2	2		2	16
3rd	−3.3	−3.6	−3.7	−3.8		2	2				2	2	2		2	12
4th	−3.9	−4.2	−4.4	−4.5		2	2				2	2	2		2	12
5th	−4.5	−4.9	−5.1	−5.3		2	2				2	2	2		2	12
6th	−5.1	−5.6	−5.8	−6.0		2	2				2	2	2		2	12
7th	−5.7	−6.3	−6.6	−6.8		2	1				2	1	2		2	10
8th	−6.3	−6.9	−7.3	−7.5							2		1		2	5
9th	−6.9	−7.6	−8.0	−8.2							2				2	4
10th	−7.5	−8.3	−8.7	−9.0							1				2	3
11th	−8.1	−9.0	−9·4	−9.7											2	2
12th	−8.7	−9.7	−10.2	−10.5											2	2
				Total	39	14	13	3	3	4	19	13	15	1	24	148

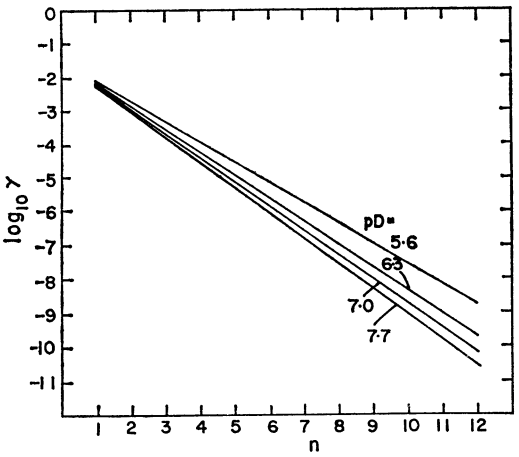

Fig. 8. $\text{Log}_{10}\ \gamma$ values assigned to the peptide NH groups located in the n-th residue from the nearer terminal of the α-helix in the myoglobin structure.

plots on the basis of this set of γ values are shown in Fig. 5 by continuous and broken lines.

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