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H_2O와 D_2O에서 메트미오글로빈의 압력에 의한 변성의 비교 연구

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Comparision of the Pressure Denaturation of Metmyoglobin in H_2O and D_2O

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요 약. 메트미오글로빈의 압력에 의한 변성의 H_2O와 D_2O에서의 차이를 pH 5.7과 pH 7.0에서 연구하였다. 메트미오글로빈은 D_2O에서 H_2O 보다 더 작은 압력에서 변성하였다. 그 차이가 pH 5.7에서가 pH 7에서 보다 더 컸다. H_2O와 D_2O에서 이 안정도의 차이가 단백질에 대한 H^+와 D^+의 결합에 차이가 있기 때문이며 또한, 수소가 중수소로 바뀜에 따르는 구조 변화 때문인 것으로 사료된다.

ABSTRACT. The stability difference of metmyoglobin in H_2O and D_2O at pH 5.7 and pH 7.0 toward pressure denaturation is studied. Metmyoglobin is denatured in D_2O at smaller pressure than in H_2O. The stability difference in H_2O and D_2O is more pronounced at pH 5.7 than at pH 7. The main reasons for the stability difference in H_2O and D_2O are the difference in positive charge due to H^+ and D^+ binding to the protein in H_2O and D_2O, and the structural change that accompany deuteration.

INTRODUCTION

The deuterium isotope effect on protein stability and structure has been investigated by several workers. Maybury and Katz[1] found that ovalbumin had a slower rate of denaturation in D_2O than in H_2O. An increase in stability toward denaturation was found by Hermans and Scheraga[2] for the thermal denaturation of ribonuclease and by Harrington and von Hippel for the melting of gelatin[3]. Berns[4] reported that phycocyanin was less stable in H_2O than in D_2O when the thermal denaturation was studied in acetate buffers (pH=4.7) but the reverse was true in phosphate buffers (pH =7.0). In a study done with poly-L-glutamic acid and poly-L-lysine in H_2O and D_2O Appel and Yang[5] found a shift of the helix-coil transition toward the alkaline side. Denaturation appeared to favor the formation of the helix for poly-L- glutamic acid, but it favored the coil for poly-L-lysine. Appel and Brown[6] studied the acid and alkaline denaturation of myoglobin in H_2O and D_2O. They found that myoglobin was less stable in D_2O than in H_2O

toward acid denaturation, but the reverse was found for alkaline denaturation. Tomita *et al*[7] found that denaturation caused extension of the α-helix and the hydrogen bonds lengthened by about 0.027Å from X-ray measurements.

These results have been variously interpreted in terms of hydrogen bonds[2,3] hydrophobic interactions[4] or difference in the dissociation constants of ionizable groups in H_2O and D_2O.

The stability difference of proteins in H_2O and D_2O toward pressure denaturation has not yet been studied. The pressure denaturation of metmyoglobin in H_2O and in D_2O were there fore investigated. The denaturation was followed by measuring the absorbance change in the Soret band (409nm) which is known to be an indicator of the denaturation protein by pressure in the acidic and neutral pH ranges[8].

EXPERIMENT

Materials. Sperm whale metmyoglobin was obtained from Sigma Chemical Co. The protein was used without further purification. The concentration of the protein in this experiment was about 3×10^{-6} *M*. D_2O (99.8 % isotopic purity) was purchased from Sigma Chemical Co., and used without purification. To avoid absorpiton of H_2O from the air, the D_2O was stored in a closed bottle in a desiccator.

Pressure Generating System and Optical Bomb. The pressure generating system and optical bomb have been described elsewhere[9].

Spectral Measurement. The protein solution was prepared by dissolving the lyophylized protein and filtering. The pH of solution was adjusted by HCl without using any buffers. pD was calculated by the relation pD=pH (apparent) +0.4[10]. In adjusting the pD of the solution, HCl ($\sim1\times10^{-4}M$) and NaOH ($\sim1\times10^{-4}$ *M*) were used. As the amount of HCl or NaOH used to adjust the pD was less than 0.5 % of total solution (one batch was around 100m*l*) and the protein in the solution ($\sim3\times10^{-6}M$) was small, this procedure would not effect the result. All of the manipulations were done in the glove box filled with N_2.

The absorbance of the protein solution was measured with the Cary 14 spectrophotometer.

RESULTS AND DISCUSSION

The change of the absorbance at the Soret band at 409nm was recorded as a function of pressure. For convenience, let the maximum absorbance at 409nm (at low pressure) in the course of this protein denaturation be assigned a value of one and the minimum absorbance (at high prssure) be assigned a value of zero; all absorbance at other pressures are expressed in terms of these reference values. We call this revalued absorbance the relative absor-

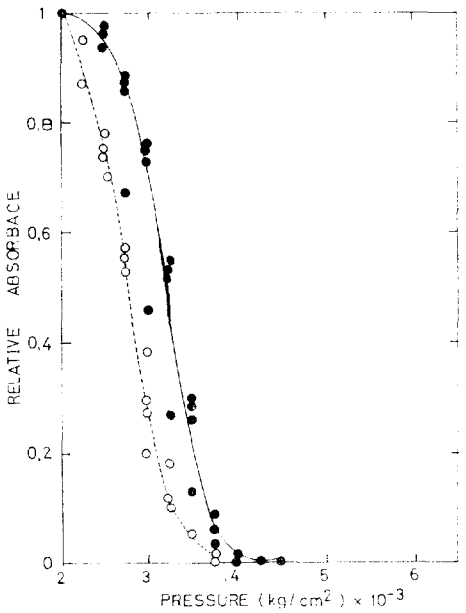


Fig. 1. The pressure denaturation of metmyoglobin in H_2O and D_2O at 5°C and pH 5.7. Thepressure denaturation in H_2O is indicated by solid lines and the pressure denaturation in D_2O is indicated by dashed lines.

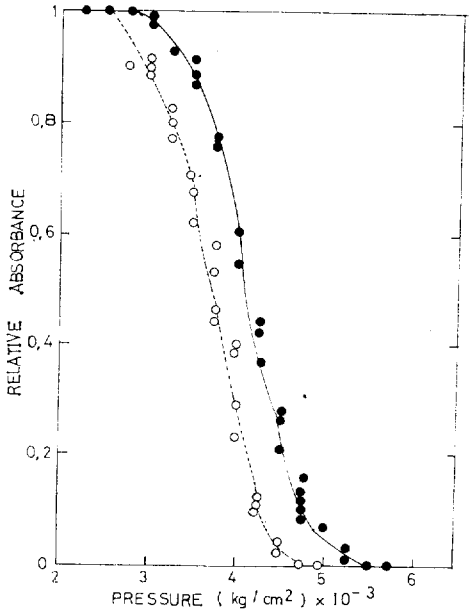


Fig. 2. The pressure denaturation of metmoglobin in D_2O at 20°C and pH 5.7. The pressure denaturation in H_2O is indicated by solid lines and the pressure denaturation in D_2O is indicated by dashed lines.

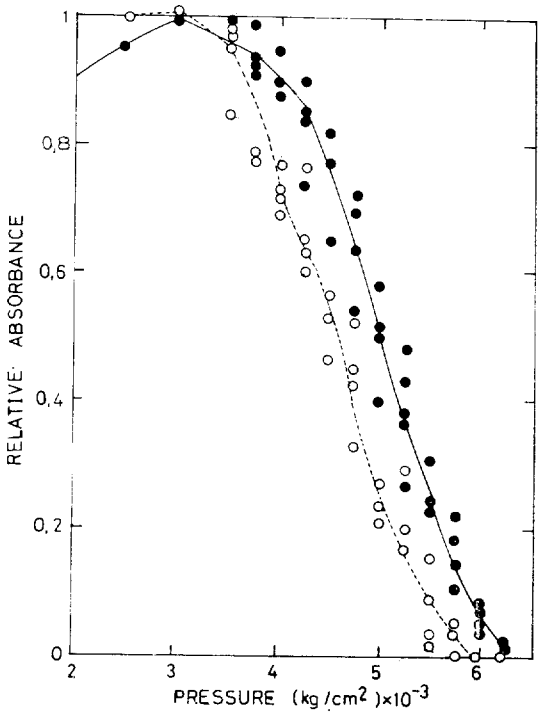


Fig. 3. The pressure denaturation of metmyoglobin in H_2O and D_2O at 40°C and pH 5.7. The pressure denaturation in H_2O is indicated by solid lines and the pressure denaturation in D_2O is indicated by dashed lines.

bance. The relative absorbance *vs.* pressure at pH 5.7 (or pD 5.7) and at 5, 20, 40°C are shown in *Figs.* 1, 2 and 3. The result at the same temparatures at pH 7 are given in *Figs.* 4, 5 and 6.

Zipp and Kauzmann[8] have shown that metmyoglobin in water denatures under pressure. We see from *Figs.* 1~6 that metmyoglobin is denatured in D_2O as well, but that the pressure required is smaller. The stability difference in H_2O and D_2O is more pronounced at pH 5.7 than at pH 7. The temperature effect on this isotope effect is barely detectable, but seems to be slightly larger at low temperatures.

Appel and Brown[6] reported that at 1 atm metmyoglobin denatures at pH 4.4 in H_2O and at pD 4.9 in D_2O. Thus metmyoglobin is more stable toward acid denaturation in H_2O than in D_2O which is in the same direction as our observations on pressure denaturation. On the other hand, several workers have found that some proteins are more stable toward thermal denaturation in D_2O than in H_2O (Hermans and Scherage[2], Harrington and von Hippel[3]). but Bernes[4] found that the relative stability toward thermal denaturation in H_2O and D_2O may depend on the pH.

These results will now be discussed in terms of hydrophobic interactions, hydrogen bonds, the weakening of the acidity of ionizable groups in D_2O and the possible bond length changs due to denaturation.

We have seen that hydrophobic interactions of small model non-polar molecules are stronger in H_2O than in D_2O (See also Kresheck *et al.*,[11] and Ben-Naim[12]), but Kresheck *et al.*[11] give reasons for believing that the hydrophobic interactions between the side chains of amino acids are stronger in D_2O than in H_2O. Our studies

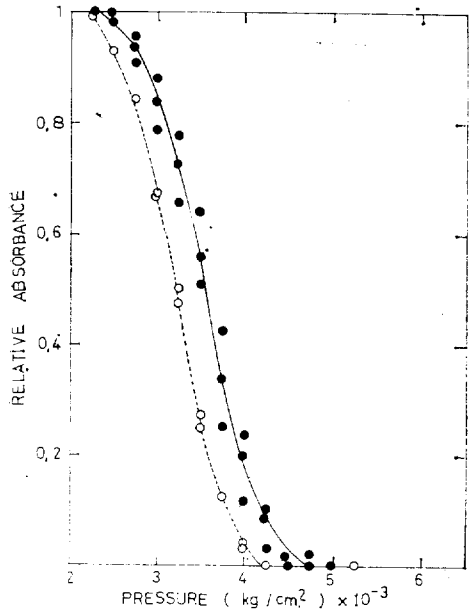


Fig. 4. The pressure denaturation of metmyoglobin in H_2O and D_2O at 5°C and pH 7. The pressure denaturation in H_2O is indicated by solid lines and the pressure denaturation in D_2O is indicated by dashed lines.

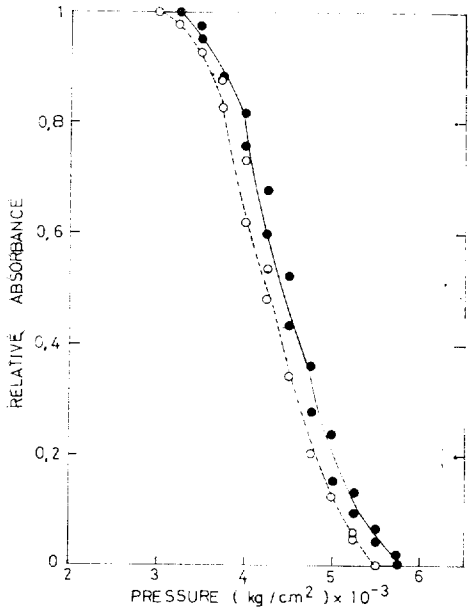


Fig. 5. The pressure denaturation of metmyoglobin in H_2O and D_2O at 20°C and pH 7. The pressure denaturation in H_2O is indicated by solid lines and the pressure denaturation in D_2O is indicated by dashed lines.

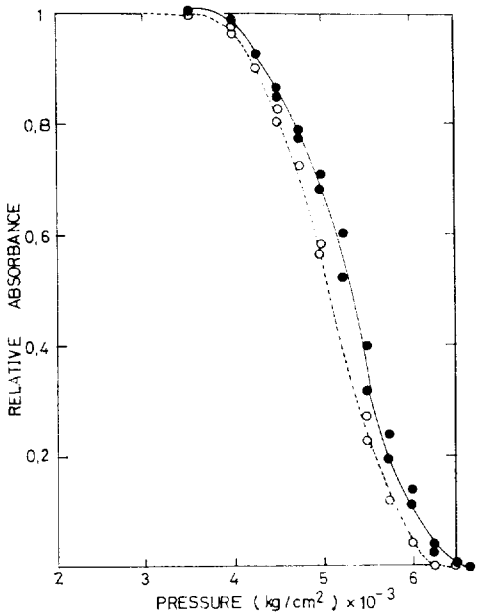
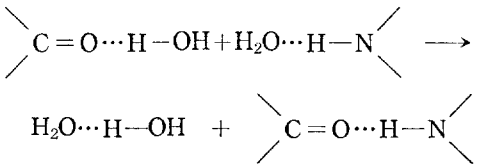


Fig. 6. The pressure denaturation of metmyoglobin in H_2O and D_2O at 40°C and pH 7. The pressure denaturation in H_2O is indicated by solid lines and the pressure denaturation in D_2O is indicated by dashed lines.

of the pressure effect on the hydrophobic interactions of 4-octanone[13] indicate that the difference between the hydrophobic interactions in H_2O and D_2O is decreased by pressure and even seems to be reversed at high pressure (above 3000 kg/cm^2. As the pressure denaturation of metmyoglobin occurs above 3000 kg/cm^2 in these experiments, the difference between the hydrophobic interactions in H_2O and D_2O is likely to contribute negligibly to the stability difference of this protein in H_2O and D_2O, if we judge by the experimental results on 4-octanone.

The helix-coil transition of the α-helix due to denaturation involves not only the breaking of hydrgen bonds of the helix, busl aso a change in the hydrogen bonding of the water molecules also involved in the transition; that is, the transition involves the reaction



Thus the deuterium isotope effect will be shown not only in the peptidepeptide hydrogen bonds, but also in the solvent-solvent and solvent-peptide interaction. As the changes in the thermodynamic properties accompanying the above reaction are small[14], a change in the hydrogen bone strengths due to deuteration would be expected to have a very small effect on the stability difference of the protein in H_2O and D_2O.

The dissociation constants of ionizable groups in H_2O are larger than in D_2O. This means that at a given pH(pD) more D^+ ions are bound by the protein in D_2O than H^+ ions in H_2O. In acid denaturation the H^+ ion can be viewed as the denaturing agent (the protein denatures more readily as it becomes more positively charged). Thus the protein will be denatured at higher pD (pH) in D_2O than in H_2O. This argument is consistent with the experimental observations of Appel and Yang[5], Appel and Brown[6] and Berns[4]. Our experimental observation that the isotope effect is less pronounced at pH 7 than at pH 5.7 can be explained by this argument: The protein is more positively charged at pH 5.7 in D_2O than in H_2O, and it is more easily denatured the higher the positive charge; therefore, it requires less pressure to denature at pH 5.7 in D_2O than in H_2O. At pH 7 the protein is relatively less positively charged than at pH 5.7, so this isotope effect is less pronounced.

It is hard to believe that the isotope effect which we see at pH 7 arises to a significant degree form isotope effects on the dissociation constants of the ionizable groups on the protein, so an additional factor must be sought which can give rise to these isotope effects. Possibly this factor is associated with the extension of the α-helix and the distortion of interatomic contacts observed on deuteration by Tomita *et al*[7]. Tomica argues that these changes make the α-helix less stable in D_2O than in H_2O. This may be the additional factor which we see operating at pH 7.

Usually, the hydrophobic interactions and bydrogen londs are important facters in the protein denaturation. But this experiment shows that these factors seem less likely to be the causes of the difference. But all of these arguments, which are based on the relatine absorbances, should be considered qualitatively not quantitatively.

CONCLUSION

We conclude that the principal reasons for the stability difference of metmyoglobin in H_2O and D_2O toward pressure denaturation are the difference in positive charge due to H^+ and D^+ binding in H_2O and D_2O and the structural changes (such as the distortion of the α-helix) that accompany deuteration. Changes in hydrophobic interactions and hydrogen bonds seem less likely to be the causes of the difference.

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