The Number of Water Molecules Bound to Insulin

Nobuya NAGASHIMA,* Ei-ichiro SUZUKI, and Masamichi TSUBOI†
Central Research Laboratories, Ajinomoto Co., Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki 210
†*Faculty of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113*
(Received September 10, 1983)

Synopsis. On cooling an aqueous solution of zinc pig-insulin to −40 °C, 108 water molecules per insulin molecule have been found (by NMR) unfrozen. This number is nearly equal to what was found crystallographically of the water molecules located within 4.7 Å of the protein molecule.

It is now well known that every globular protein is biologically active only when it is hydrated. Thus, the water molecules bound to a protein molecule are considered to play an essential role in its biological function. Their molecular structures as found in the crystal also involve a great amount of water, and these structures has been supposed to be kept on going from crystals to aqueous solutions. As a powerful tool for characterizing such protein-bound water, we have recently developed[1] a full computerized apparatus, whose main part is a broad-line pulsed NMR spectrometer with a Lamor frequency of 20 MHz, for the observation of a proton (Bruker, minispec p20). In this apparatus, the in-put is an aqueous solution of the sample protein now in question, while the out-put is the unfrozen-water content *versus* the temperature curve, where unfrozen water is defined as water molecules with protons of T_2>70 μs (here, T_2 is the spin-spin relaxation time). At any temperature below 0 °C, the usual solvent water is frozen, and the T_2 values for such water molecules are much shorter than 10 μs. Only some H_2O molecules in a special environment, such as hydrating water of a protein molecule, may be kept unfrozen, may keep a long T_2, and may be detected (as unfrozen water) by the present apparatus. The purpose of this paper is to compare what is found with this apparatus and what is found by an X-ray crystallographic study with regard to the hydration of insulin.

The sample of Zn pig-insulin powder was kindly provided by Dr. Noriyoshi Sakabe, Nagoya University; it is the same as what was subjected to his and his collaborators' crystallographic study.[2-4] Elementary analyses for this powder gave: C, 47.24; H, 6.80; N, 13.93%. This is consistent with the chemical formula of $C_{256}H_{383}N_{66}O_{76}S_6 \cdot (1/3)Zn \cdot 28H_2O$, so the water content of this powder was estimated to be 8.0 ±1.0%. This means that dry matter (DM) content=92.0 ±1.0%. The water content was also determined by measuring the weight decrease on keeping the powder sample at 115 °C for 8 h. This gave a value of H_2O content=9.4±1.0% (dry matter content=90.6 ±1.0%). A 29.2-mg portion of this powder (which should involve 26.9 mg of dry matter) was dissolved into 0.22 ml of distilled water, and this solution was then placed at the proper position in our apparatus. The out-put is illustrated in Figs. 1 and 2. As may be seen in Fig. 1, the unfrozen-water content drops suddenly at −1.5 °C (after some supercooling) in the process of cooling; this corresponds to the freezing of the solvent water. On further cooling, the unfrozen-water content is gradually lowered from 1.3 to 0.4 gH_2O/gDM. After it reaches 0.33_8 gH_2O/gDM at −25 °C, it remains nearly constant until −50 °C. In the course of warming, this unfrozen-water content is kept till the temperature becomes −10 °C, whereupon it suddenly increases. Thus, the unfrozen-water content 0.33_8 gH_2O/gDM at −40 °C seems to have some special significance; this unfrozen-water content corresponds to 108 H_2O molecules/insulin molecule, and to 216 H_2O molecules/two insulin molecules (see Table 1).

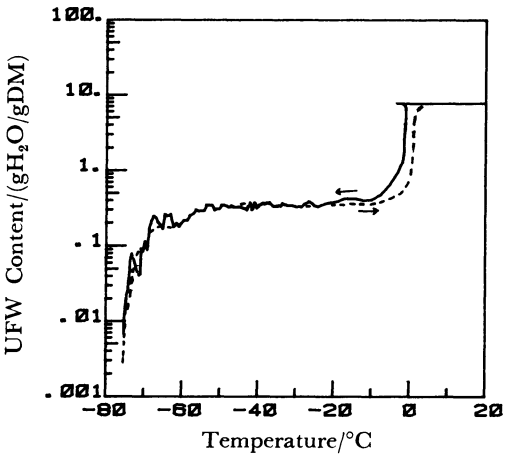

Fig. 1. Unfrozen water content *versus* temperature curves for zinc pig-insulin solution. The unfrozen water (UFW) content is given by g H_2O per g DM (dry matter). Solid line was obtained in the process of cooling (freezing curve), and dashed line was obtained in the process of warming (thawing curve).

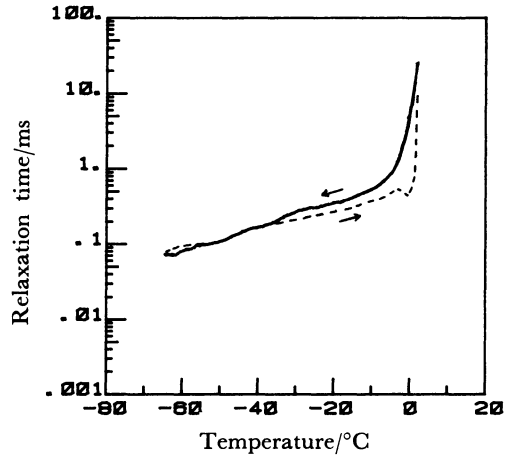

Fig. 2. T_2 (spin-spin relaxation time) *versus* temperature curves for zinc pig-insulin solution. Solid line was obtained in the process of cooling, and dashed line was obtained in the process of warming.

Table 1. Number of water molecules bound to the pig insulin molecule

X-Ray crystallography[a]					NMR (Present work)		
Nearest atom in insulin	Distance / Å	Number per asymmetric unit		Temperature range in which the water is kept unfrozen / °C	T_2 / ms	UFW content / gH_2O/gDM	Number per two insulin molecules
N or O	2.7—3.3	117	124	−50——25	0.1—0.3	0.33_8	216
C	3.2	7					
N or O	3.3—4.7	43	90				
C	3.3—4.7	47					
			214				
N or O	4.7—5.0	5	11	−25——8	0.3—0.5	0.10_0	64
C	4.7—5.0	6					
			66				
Not found		55[b]					

a) Refs. 2—4. b) This may be greater than 55, because the total number of water molecules in the crystal was determined by chemical analysis, while the located molecules may have occupancy factors with values slightly less than 1.0 (private communication from Dr. N. Sakabe).

For the rhombohedral two-zinc porcine insulin crystal, a high-resolution (1.2 Å) X-ray diffraction analysis has been made.[2-4] A chemical analysis indicated that its asymmetric unit involves 2/3 Zn+2 insulin molecules+280 H_2O. Of these 280 H_2O, 225 were crystallographically determined. Among them, 214 H_2O molecules were found within the distance of 4.7 Å from the nearest atoms of insulin, and 11 H_2O molecules at distances of 4.7—5.0 Å from the nearest atoms. The remaining 55 H_2O molecules were not found because of their disorder and the larger temperature factors (see Table 1). Therefore, the water molecules remaining unfrozen in the −50—−25 °C range (or in the −50—−10 °C range in the thawing curve; see Fig. 1) may be correlated with the water of hydration located within 4.7 Å of the protein molecule. This may be called "tightly bound" water. The remaining 66 H_2O molecules were found in the crystallographic work to be less strongly bound to the insulin molecules. This number corresponds to the amount of water kept unfrozen in the temperature range of −8—−25 °C on the freezing curve (or −15—−3 °C on the thawing curve) in our NMR study. This may be called "weakly bound" water. In view of the errors (about ±5%) in our unfrozen-water content estimation, it is also possible that all the crystallographically located (at 4 °C) H_2O molecules (225 in number) correspond to unfrozen water at −40 °C ("tightly bound" water). If so, all the 55 water molecules that do not appear clearly in the crystal correspond to the "weakly bound" water.

The above-mentioned apparatus can also provide an average T_2 value of the unfrozen water at every temperature.[1] As is shown in Fig. 2, the "tightly bound" water was found to have a T_2 value of 0.1—0.3 ms, and the "weakly bound" water, one of 0.3—0.5 ms. It should also be pointed out here that a hysteresis loop in the freezing-thawing curves is found not only for the unfrozen water content (see Fig. 1), but also for the unfrozen water mobility (Fig. 2). By thermal analysis with a differential scanning calorimeter (Daini Seikosha, SSC 560U), an inflection was observed in the thawing process at −32.8 °C; this means an increase in the specific heat.

In conclusion, it may be suggested that the tightly-bound water molecules (and possibly the weakly-bound water molecules also) are nearly intrinsic on the surface of the insulin molecule, and that even in its aqueous solution their sites and orientations are similar to those in the crystal. This suggestion is based merely upon a coincidence in the numbers. If it is valid, however, it may be generalized to globular proteins other than insulin.

We wish to express our thanks to Dr. Noriyoshi Sakabe, Nagoya University, for his kindness in providing the sample and for his valuable advice.

References

1) E. Suzuki and N. Nagashima, *Bull. Chem. Soc. Jpn.*, **55**, 2730 (1982).

2) N. Sakabe, K. Sakabe, and K. Sasaki, "Proc. Symp. on Proinsulin, Insulin, and C-Peptide," (Tokushima, 12—14 July 1978), ed by S. Baba, T. Kaneko, and N. Yanaihara, Excerpta Medica, Amsterdam (1978), p. 73.

3) K. Sakabe, N. Sakabe, and K. Sasaki, "Water and Metal Cations in Biological Systems," ed by B. Pullman and K. Yagi, Japan Scientific Societies Press, Tokyo (1980), p.117.

4) N. Sakabe, K. Sakabe, and K. Sasaki, "Structure Studies on Molecules of Biological Interest," ed by G. Dodson, J. P. Glusker, and D. Sayre, Clarendon Press, Oxford (1981), p.509.