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Effect of Solute Hydrogen Bonding Capacity on Osmotic Stability of Lysosomes

Shu-Jing Hao[1,2], Jun-Fang Hou[3], and Guo-Jiang Zhang[1]*

[1]*Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, PR China; E-mail: zhanggj@sun5.ibp.ac.cn*
[2]*Graduate School, Chinese Academy of Sciences, Beijing 100039, PR China*
[3]*School of Physical Science and Technology Sichuan University, Chengdu 610064, PR China*

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Abstract—The effect of solute hydrogen bonding capacity on the osmotic stability of lysosomes was examined through measurement of free enzyme activity of lysosomes after their incubation in sucrose and poly(ethylene glycol) (PEG) (1500-6000 Da molecular mass) media. Free enzyme activity of the lysosomes was less in the PEG medium than that in the sucrose medium under the same hypotonic condition. The lysosomal enzyme latency loss decreased with increasing hydrogen bonding capacity of the solute. In addition, the lysosomes lost less latency at lower incubation temperature. The results indicate that solute hydrogen bonding capacity plays an important role in the osmotic protection of an incubation medium to lysosomes.

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Key words: hydrogen bonding capacity, lysosomal osmotic stability, lysosome

Lysosomes are called intracellular osmometers because the organelle is sensitive to osmotic stress [1]. Osmotic imbalance across the membranes can destabilize lysosomes. Generally, isolated lysosomes are suspended in isotonic sucrose medium or other isotonic medium. The solutes of the medium must be impermeable to the lysosomal membranes. Thus, the medium maintains osmotic balance across the lysosomal membranes and protects their integrity. The lysosomal membranes are permeable to various molecules and ions. The entry of solutes into lysosomes causes osmotic imbalance across the membranes, which is followed by the influx of water into the organelle. It eventually leads to the disruption of the lysosomes. As demonstrated previously, the hydrogen bonding capacity of a solute greatly affected its osmotic protection effect on suspended lysosomes [2]. The study shows that solutes with large hydrogen bonding capacity cannot enter the lysosomes. Therefore, the lysosomes can maintain their integrity. As proposed previously, the hydrogen bonding capacity of a molecule is an indicator of the difficulty it will experience in leaving an aqueous milieu for the hydrophobic environment of a phospholipid bilayer [3]. This explains why it is difficult for a solute with large hydrogen bonding capacity to leave aqueous milieu for the lysosome interior and why the solute can produce better osmotic protection to the lysosomes. The hydrogen bonds between solutes and water can prevent leaving of solute from the aqueous milieu. It is of interest to elucidate whether a solute can drag water through a hydrogen bond and therefore prevent water from leaving the aqueous milieu. The results of this study show that greater hydrogen bonding capacity of solutes is unfavorable for water to leave the incubation medium and enter lysosomes.

Abbreviations: PEG, poly(ethylene glycol).

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials. 4-Methylumbelliferyl-N-acetyl-β-D-glucosaminide was from Sigma (USA). Percoll was purchased from Amersham (Sweden). Other chemicals were of analytical grade from Beijing Chemical Factory (China).

Preparation of lysosomes. A male Wistar rat was starved for 24 h and killed by decapitation. Liver lysosomes were isolated as described previously [4]. Briefly, the rat liver was homogenized in 0.25 M sucrose and centrifuged at 3000*g* for 8 min. The supernatant was incubated at 37°C for 5 min in the presence of 1 mM $CaCl_2$ to

promote separation of lysosomes from mitochondria [5]. Then, the supernatant was centrifuged at 20,000*g* for 20 min. The pellet was resuspended in sucrose solution, mixed with Percoll (2 : 1 v/v), and centrifuged at 40,000*g* for 90 min. The lower 1/4 volume of the gradient (lysosomal fraction) was pooled, mixed with 10 volumes of 0.25 M sucrose, and centrifuged at 10,000*g* for 13 min to remove Percoll. The purified lysosomes were resuspended in 0.25 M sucrose medium at 1.35 mg membrane protein per milliliter for use. All steps were performed at 4°C.

Assay of lysosomal integrity. Lysosomal integrity was assessed by measuring lysosomal marker enzyme latency. The enzyme latency refers to the percent of intact lysosomes as revealed by the inability of substrate to reach the lysosomal enzyme before the organelles are deliberately ruptured [6]. 4-Methylumbelliferyl-N-acetyl-β-D-glucosaminide, the substrate of lysosomal β-hexosaminidase, was used at 1 mM to measure the enzyme activity [7]. The liberated 4-methylumbelliferone was determined by measuring its fluorescence (excitation 365 nm, emission 444 nm) on a Hitachi F-4010 (Japan) fluorescence spectrophotometer. Activities of the enzyme measured in the absence and presence of 0.36% Triton X-100 was designated the free activity and the total activity, respectively. Loss of lysosomal integrity was determined as increased percentage of free activity.

Assay of lysosomal osmotic stability. The osmotic stability of lysosomes was assessed by examining their integrity after incubation in hypotonic medium. Lysosomal sample (20 μl) was incubated with 200 μl of hypotonic sucrose or poly(ethylene glycol) (PEG) medium at 25 or 37°C for the indicated time. After the incubation, a 50-μl portion of the lysosomal suspension was used for the assay of lysosomal integrity. Increase in the free enzyme activity of lysosomes indicated loss of the lysosomal osmotic sensitivity. Osmolality of each medium was measured using a Vapro 5520 vapor pressure osmometer.

Calculation of hydrogen bonding capacity of molecules. As described previously, the calculation of hydrogen bonding capacity of molecules assigns a hydrogen bonding capacity to each functional group (e.g. an aliphatic OH group = 2; C–O–C = 0.8; CO_2–C = 0.8), and the total for a molecule is a simple sum of the parts [8]. Thus, sucrose is assigned a hydrogen bonding capacity of 18.4. The hydrogen bonding capacity of PEG is calculated according to its chemical structure, which shows repeating ethylene oxide moieties [2]:

$$HO\text{–}\left[\text{–}CH_2\text{–}CH_2\text{–}O\text{–}\right]_n H.$$

RESULTS

In this study, lysosomes were incubated in sucrose and PEG 1500-6000 media. As shown in Table 1, the hydrogen bonding capacity of PEG molecules (molecule weight ≥1500) is greater than that of the sucrose molecule and the hydrogen bonding capacity of PEG molecules increases with increasing molecular mass.

Table 1. Hydrogen bonding capacity of solute molecules

Solute	Hydrogen bonding capacity
Sucrose	18.4
PEG 1500	30.1
PEG 4000	75.6
PEG 6000	112.0

As reported by Lloyd and Forster [1], lysosomes can maintain their integrity in isotonic medium that contains an impermeable solute. This means that an impermeable solute can provide perfect osmotic protection to lysosomes under isotonic conditions. To study the effect of hydrogen bonding capacity of solute molecules on the osmotic stability of lysosomes, we incubated lysosomes in hypotonic sucrose and PEG 6000 medium and compared the lysosomal free enzyme activity in the two media. As shown in Table 2, free enzyme activity of the lysosomes is higher in the sucrose medium than in the PEG 6000 medium under each hypotonic condition (osmolality at 120, 140, and 160 mmol/kg). The lysosomal enzyme latency loss is less in the PEG 6000 medium than that in the sucrose medium at each osmolality, indicating that the PEG 6000 medium provided better osmotic protection to the lysosomes. As shown in Table 2, the hydrogen bonding capacity of PEG 6000 is greater than that of sucrose at each osmolality. This suggests that greater hydrogen bonding capacity is unfavorable for water to leave the PEG 6000 medium for the interior of lysosomes.

To further study the effect of solute hydrogen bonding capacity on lysosomal osmotic stability, we incubated lysosomes in hypotonic (137 mmol/kg) sucrose, PEG 1500, PEG 4000, and PEG 6000 media, and then measured the lysosomal free enzyme activity. Previous study has established that the lysosomal membranes are impermeable to sucrose and PEG molecules with molecule mass above 1000 Da [2]. As shown in Table 3, free enzyme activity of the lysosomes decreased from 49 to 30% with increasing the solute hydrogen bonding capacity. In this experiment, the lysosomes suffered the same hypotonic pressure in each incubation medium. Because the solutes cannot enter the lysosomes, the lysosomal latency loss should be similar after incubation in these media. In other words, the same hypotonic pressure of these media should cause the same level of the lysosomal latency loss. However, the lysosomal latency increased with decreasing solute hydrogen bonding capacity. The result suggests that

Table 2. Effects of medium osmolality and solute hydrogen bonding capacity on lysosomal free enzyme activity

Osmolality of incubation medium, mmol/kg	Hydrogen bonding capacity*, mmol/kg		Free hexosaminidase activity**, %	
	sucrose	PEG 6000	sucrose	PEG 6000
120	1321	1971	80.4 ± 0.7	55 ± 1
140	1748	2352	48.0 ± 0.4	29.3 ± 0.3
160	2326	2688	27.0 ± 0.2	20.0 ± 0.6

* Lysosomal samples were incubated in sucrose or PEG 6000 medium at 25°C for 5 min. Hydrogen bonding capacity of each medium is calculated as the hydrogen bonding capacity of the solute molecule multiplied by solute concentration.
** Values are means ± S.D. of three measurements. Statistical analysis was performed using Student's *t*-test.

the hydrogen bonding capacity of solute molecules plays an important role in the osmotic protection of the solute to lysosomes.

As demonstrated previously, hydrogen bonding is temperature dependent. When temperature increases, the hydrogen bond stability decreases [9]. To verify whether the hydrogen bonding capacity of a solute affects the osmotic stability of lysosomes, we incubated the lysosomes in hypotonic sucrose and PEG media at 25 and 37°C, respectively. Then we compared the lysosomal latency loss at the two temperatures. As shown in Table 4, free enzyme activity of the lysosomes is less at 25°C than at 37°C for the three incubation media. Compared with sucrose medium (lysosomal free enzyme activity is 53% at 25°C and 57% at 37°C), the PEG 6000 medium provided better osmotic protection at 25°C (free enzyme activity 33%) than that at 37°C (54%). To clarify whether the lysosomes are more prone to disruption at 37°C than at 25°C regardless of the solute hydrogen bond, we repeated the above experiment in K_2SO_4 medium. The lysosomes were incubated in the K_2SO_4 medium with the same hypotonic osmolality (132 mmol/kg) at 25 and 37°C, respectively, for 10 min. The result shows that free activity of the lysosomes is 57.0% at 25°C and 57.3% at 37°C. The lysosomes lost similar latency at the two different temperatures in the K_2SO_4 medium. This indicates that the higher temperature (37°C) itself did not increase the lysosomal latency loss. K_2SO_4 cannot form hydrogen bonds with water. These results suggest that the hydrogen bonds of the solute caused decrease in the lysosomal latency loss in the PEG 6000 medium at 25°C. The PEG 6000 molecules possess greater hydrogen bonding capacity than the sucrose molecules. The lower temperature (25°C) improved the osmotic protection of PEG 6000 medium to the lysosomes more markedly than that of the sucrose medium, presumably due to the greater stabilizing effect of the low temperature on the hydrogen bonds of PEG 6000 molecules. Thus, the PEG 6000 medium can better protect the lysosomes. The results further establish that the hydrogen bonding capacity of the

Table 3. Effect of solute hydrogen bonding capacity on lysosomal free enzyme activity

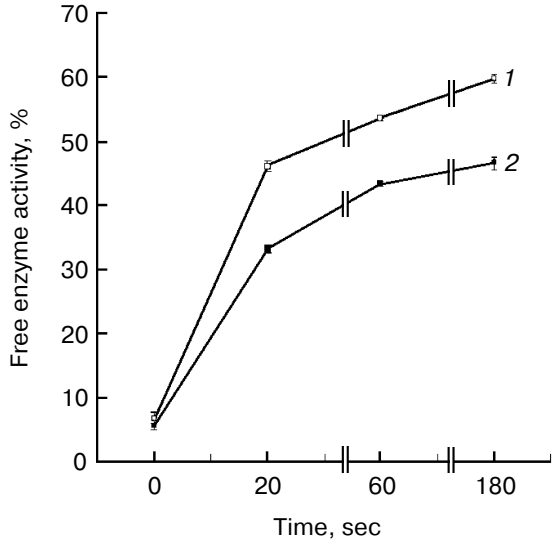
Solute*	Hydrogen bonding capacity, mmol/kg	Free hexosaminidase activity**, %	*p*
Sucrose	1715	49.7 ± 0.8	–
PEG 1500	1806	40.6 ± 1.1	<0.001
PEG 4000	2124	35.6 ± 0.2	<0.001
PEG 6000	2330	30.8 ± 0.7	<0.001

* Sucrose, PEG 1500, PEG 4000, and PEG 6000 were prepared as hypotonic solutions at 137 mmol/kg osmolality. Lysosomal samples were incubated in each medium at 25°C for 5 min.
** Values are means ± S.D. of four measurements. Statistical analysis was performed using Student's *t*-test.

Table 4. Effects of temperature and solute hydrogen bonding capacity on lysosomal free enzyme activity

Solute*	Hydrogen bonding capacity, mmol/kg	Free hexosaminidase activity**, %	
		25°C	37°C
K_2SO_4	0	57.0 ± 1.1	57.3 ± 1.1
Sucrose	1656	53.3 ± 1.0	57.3 ± 0.5
PEG 1500	1764	44.9 ± 1.9	56.2 ± 0.9
PEG 6000	2274	33.7 ± 0.9	54.9 ± 0.5

* K_2SO_4, sucrose, PEG 1500, and PEG 6000 were prepared as hypotonic solutions at 132 mmol/kg osmolality. Lysosomal samples were incubated in the medium at 25 or 37°C for 10 min.
** Values are means ± S.D. of three measurements.



Effect of incubation duration on free enzyme activity of lysosomes. Lysosomal samples were incubated in sucrose or PEG 6000 medium at 25°C for the indicated times. Osmolality of both media was 126 mmol/kg. Values are means ± S.D. (n = 3). *1*) Sucrose; *2*) PEG 6000

solute molecules affects their osmotic protection of lysosomes.

As shown previously, an osmotic imbalance across the lysosomal membranes can drive water flow into the lysosomes and osmotically disrupt the lysosomes [1]. Knowing that the greater hydrogen bonding capacity of solutes can promote their osmotic protection to lysosomes, we examined whether the hydrogen bonding capacity of solutes affect the influx of water into lysosomes. As shown in the figure, the lysosomes were incubated in the hypotonic (126 mmol/kg) sucrose and PEG 6000 medium for 20, 60, and 180 sec. Then, the free enzyme activity was measured. Since the lysosomes were incubated in the media with the same hypotonic osmolality, the amount of water flowing into the lysosomes and the lysosomal latency loss should be similar. However, the free enzyme activity of lysosomes increased more markedly and rapidly in the sucrose medium than that in the PEG 6000 medium. The results indicate that water flowed into the lysosomes more rapidly and greatly in the sucrose medium than that in the PEG 6000 medium.

DISCUSSION

Lysosomes are sensitive to osmotic stress. Osmotic imbalance across their membranes can disrupt the organelle through an influx of water. In this study, we suspended lysosomes in hypotonic medium. The media contained different solutes but possessed the same osmolality. The same hypotonic pressure of the media should drive a similar amount of water into the lysosomes, causing a similar level of the lysosomal latency loss. However, the free enzyme activity of the lysosomes was different under the same hypotonic condition. This means that the extent of water influx was different in the media with the same hypotonic osmolality. As shown above, the effects of the incubation medium on the lysosomal stability correlated with the hydrogen bonding capacity of the solutes. The lysosomal latency loss decreased with increasing solute hydrogen bonding capacity. This correlation is prominent at greater hydrogen bonding capacity of the solutes. As shown in Table 4, only the PEG medium with greater hydrogen bonding capacity provided good osmotic protections to the lysosomes. This suggests that greater solute hydrogen bonding capacity cam prevent water leaving the incubation medium for the lysosomal interior. Biological experiments must be carried out in isotonic medium to protect the integrity of living cells and isolated organelles. In this study, we demonstrated that the hydrogen bonding capacity of a solute is another important property of the incubation medium. It can affect the stability of the incubated cells and organelles. This conclusion can have significance for biological studies. Hydrogen bond is a noncovalent, attractive interaction between a proton donor X–H and a proton acceptor Y in the same or in a different molecule. There are aqueous solutions in which the hydrogen bonds between solute and water are so strong and numerous that the mixtures are thermodynamically very stable. The substances in this category include sucrose and polyalcohols [10]. As proposed previously, some solutes can drag solvents through hydrogen bonds [11]. This dragging force is unfavorable for the solutes and solvents to leave the solution. A recent study established that the hydrogen bonds between polymer and water strongly depress the diffusion of water molecules [12]. This view is supported by the evidence that hydrogen bonds can prevent osmotic water flowing across the bladder wall and the fluidity of water in bulk solution [13]. The hydrogen bonds between solutes and water can also prevent leaving of the solute from the aqueous milieu [3]. Polar solute molecules, when dissolved in water, form very strong hydrogen bonds with the solvent. Such molecules need to break the hydrogen bonds to enter another phase [14]. The evidence can explain why the increase in the hydrogen bonding capacity of the solutes can improve their osmotic protection to the lysosomes. It is well known that the electrostatic energy of water is much lower in aqueous medium with high dielectric constant (about 80) than in a typical bilayer with low dielectric constant (about 2) [15]. For permeation of water across membranes by the solubility–diffusion mechanism, Born energy is required to counteract the energy barrier and transfer water from the high-dielectric aqueous phase to the low-dielectric membrane interior [16]. It has been established that hydrogen bonds can increase the dielec-

tric constant of a medium [17, 18]. As a result, increased number of hydrogen bonds can decrease electrostatic energy of a medium through increase in its dielectric constant. It thus increases the energy barrier for the permeation of water across membranes. In other words, more energy is required for water to cross a membrane when the hydrogen bonds of the medium are increased. This explains why the lysosomes were more stable in the medium with greater solute hydrogen bonding capacity.

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REFERENCES

1. Lloyd, J. B., and Forster, S. (1986) *Trends Biol. Sci.*, **11**, 365-368.
2. Iveson, G. P., Bird, S. J., and Lloyd, J. B. (1989) *Biochem. J.*, **261**, 451-456.
3. Lloyd, J. B. (1992) in *Pathophysiology of Lysosomal Transport* (Thoene, J. G., ed.) Chap. 7, CRC Press, Boca Raton, pp. 295-308.
4. Jonas, A. J., Smith, M. L., Allison, W. S., Laikind, P. K., Greene, A. A., and Schneider, J. A. (1983) *J. Biol. Chem.*, **258**, 11727-11730.
5. Yamada, H., Hayashi, H., and Natori, Y. (1984) *J. Biochem.*, **95**, 1155-1160.
6. Greene, A. A., and Schneider, J. S. (1992) in *Pathophysiology of Lysosomal Transport* (Thoene, J. G., ed.) Chap. 2, CRC Press, Boca Raton, pp. 7-44.
7. Bird, S. J., Forster, S., and Lloyd, J. B. (1987) *Biochem. J.*, **245**, 929-931.
8. Diamond, J. M., and Wright, E. M. (1969) *Annu. Rev. Physiol.*, **31**, 581-646.
9. Ross, P. D., and Rekharsky, M. V. (1996) *Biophys. J.*, **71**, 2144-2154.
10. Rowlinson, J. S. (1957) in *Hydrogen Bonding* (Hadzi, D., ed.) Pergamon Press, London, pp. 423-427.
11. Hammel, H. T., and Schlegel, W. M. (2005) *Cell Biochem. Biophys.*, **42**, 277-345.
12. Zhao, Z. J., Wang, Q., Zhang, L., and Wu, T. (2008) *J. Phys. Chem. B*, **112**, 7515-7521.
13. Eggena, P. (1983) *Am. J. Physiol.*, **244**, C44-9.
14. Avdeef, A., and Tsinman, O. (2006) *Eur. J. Pharm. Sci.*, **28**, 43-50.
15. Orme, F. W., Moronne, M. M., and Macey, R. I. (1988) *J. Membr. Biol.*, **104**, 57-68.
16. Deamer, D. W., and Bramhall, J. (1986) *Chem. Phys. Lipids*, **40**, 167-188.
17. Sharma, M., Resta, R., and Car, R. (2007) *Rev. Lett.*, **98**, 247401.
18. Yoshii, N., Miura, S., and Okazaki, S. (2001) *Lett.*, **345**, 195-200.