EFFECT OF TEMPERATURE ON HAEMAGGLUTINATING ACTIVITY AND ON THE CONFORMATION OF LEUCOAGGLUTININ, A LECTIN FROM *PHASEOLUS VULGARIS* (RED KIDNEY BEAN)

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1. Introduction

One of the isolectins from *Phaseolus vulgaris* (red kidney bean) is called leucoagglutinin because of its high leucoagglutinating activity [1]. This lectin consists of 4 identical subunits (L-subunit) which bind, with high affinity, glycidic receptors on lymphocyte surface membranes [2–3]. It has been shown that leucoagglutinin interacts specifically with the glycomoiety of human Tamm-Horsfall glycoprotein [4] and that the glycopeptide purified after pronase digestion of the glycoprotein is a powerful inhibitory hapten of the precipitin reaction between leucoagglutinin and Tann-Horsfall glycoprotein [5]. Leucoagglutinin is also able to agglutinate neuraminidase-treated human erythrocytes and agglutination is inhibited by very low concentration (3.2 μM) of the glycopeptide isolated from human Tamm-Horsfall glycoprotein (T-H-glycopeptide) [6].

Studies on the haemagglutinating activity of different lectins have shown that the rate of agglutination may be affected by temperature [7]. The erythrocyte agglutination by concanavalin A (con A) is reduced at low temperature [8]. This difference has been related to changes in the molecular conformation of the lectin [9,10].

This communication presents data on the agglutination of human desialylated erythrocytes by leucoagglutinin at different temperatures. The temperature-dependent changes of leucoagglutinin conjugated with fluorescent compounds were also investigated by the method of fluorescence polarization. The results obtained suggest that differences in the haemagglutinating activity of leucoagglutinin may be related to a change of the conformation of the molecule, bound to the glycidic receptors, induced by temperature.

2. Experimental

2.1. *Materials*

Leucoagglutinin and other lectins were from Pharmacia. The conjugate of leucoagglutinin with dansyl chloride (DNS Sigma) was prepared at room temperature. To 2 mg leucoagglutinin in 1 ml 0.1 M $NaHCO_3$, containing 0.1 M *N*-acetylgalactosamine, were added 30 mg DNS-cellulose powder prepared as in [11]. The suspension was stirred for 30 min and cellulose was removed by centrifugation. The dansylated lectin was separated from low M_r reagents by gel-filtration on Sephadex G-25 equilibrated and eluted with PBS (20 mM sodium phosphate buffer (pH 7.4) containing 0.14 M NaCl). The fluorescent fractions emerging with the void volume were used for fluorescent polarization measurement. The protein/dye ratio was calculated from A_{280} and A_{335} measurements ($E^{1\%}_{280}$ 11.4; ϵ_{335} 3.3 × 10^3); the ratio was 0.26.

Fluoresceination of leucoagglutinin was performed on a solution of lectin at 1 mg/ml dialysed against 0.025 M (pH 9) Na_2CO_3. To 5 ml of this solution, brought to 1 M with NaCl, 0.5 mg fluorescein isothiocianate (Sigma) dissolved in 50 μl acetone were added; to protect the carbohydrate binding site, 0.1 M *N*-acetyl-galactosamine was present in the reaction mixture. The reaction was allowed to proceed for 24 h at 4°C. During this period the pH was controlled and, if required, adjusted to pH 9. The conjugated lectin was purified by gel-filtration as above. The protein/dye ratio was 0.96 ($E^{1\%}_{280}$ 11.4; ϵ_{493} 73 × 10^3).

T-H-glycopeptide was purified from human Tamm-Horsfall glycoprotein as in [5].

2.2. *Haemagglutination assay of desialylated erythrocytes*

Neuraminidase-treatment of human erythrocytes was performed for 45 min as in [6]. The haemagglutination assay in [6] was used. All solutions and the suspension of erythrocytes were equilibrated at the designed temperature before agglutination.

2.3. *Fluorescence polarization measurement*

The measurement of polarization at different temperatures was performed on samples of leucoagglutinin conjugated either with DNS or with fluorescein. The theoretical basis, definition of terms and equations used to elaborate experimental data were those in [11]. An Aminco-Bowman spectrophotometer equipped with Polaroid filter HNP' B was used. Polarization was measured over 0°–37°C. Temperature and other conditions were controlled as detailed in [11]. For dansylated leucoagglutinin excitation and emission wavelengths were 335 and 500 nm, respectively. For dansyl chloride a value of τ (the lifetime of the excited state of the fluorofore) of 1.2×10^{-8} s was introduced according to [12]. The wavelengths of excitation and emission of fluoresceinated lectin were 493 and 520 nm, respectively, and the value of $\tau 5 \times 10^{-9}$ s for fluorescein was assumed [12]. In all calculations the viscosity of water was assumed for the solvent.

3. Results

3.1. *Haemagglutination test*

Table 1 shows the haemagglutinating activity of leucoagglutinin tested at different temperatures. The activity of other lectins is included. The agglutination assay was in all cases performed with neuraminidase-treated erythrocytes. While no temperature dependence was observed in the agglutinating activity of soybean, wheat germ and *Lens culinaris* agglutinin, leucoagglutinin showed an activity 30-fold higher at 4°C than at 37°C.

Table 1
Average lowest lectin concentrations causing agglutination of human desialylated erythrocytes at three different temperatures

Lectin	Lowest lectin concentration (μg/ml)		
	4°C	22°C	37°C
Leucoagglutinin	3	12	90
Lens culinaris agglutinin	3	3	3
Soybean agglutinin	1	1	1
Wheat germ agglutinin	4	4	4

Haemagglutination was performed in microtiter plates. The erythrocytes (1.2%, v/v) were of group A

The inhibition of agglutination induced by glycidic haptens is considered a valuable index of their binding to lectins [13]. We tested the ability of T-H-glycopeptide to inhibit the haemagglutination induced by leucoagglutinin at various temperatures. Table 2 shows the minimal concentrations of T-H-glycopeptide required to inhibit the agglutination at 4°C, 22°C and 37°C, respectively, and its molar ratios in respect to the leucoagglutinin present in the agglutination test at each temperature. It is evident that at 37°C the glycopeptide is a good inhibitor; this result does not indicate a decrease in the affinity of the ligand for leucoagglutinin at higher temperatures.

No difference in the behaviour of leucoagglutinin was observed after conjugation with DNS or fluorescein, neither in the agglutination nor in the hapten inhibition test. The haemagglutination induced by con A, soybean, wheat germ and *Lens culinaris* agglutinins was not inhibited by T-H-glycopeptide (50 μM) (not shown), a phenomenon confirming the specificity of T-H-glycopeptide for leucoagglutinin.

3.2. *Fluorescence polarization*

Fluorescence polarization of dansylated or fluoresceinated leucoagglutinin was measured in the absence

Table 2
Inhibitory power of T-H-glycopeptide on the haemagglutination induced by leucoagglutinin at three different temperatures

Temp. (°C)	Leucoagglutinin (μM)	T-H-glycopeptide needed for inhibition (μM)	Glycopeptide / leucoagglutinin
4	0.05	1.6	32
22	0.2	3.2	16
37	1.5	13	8.5

At each temperature the concentration of leucoagglutinin was twice the minimal which induced agglutination at that temperature (see table 1). The last column is the ratio between the minimal concentration of T-H-glycopeptide required in each set for inhibition and the concentration of leucoagglutinin present. The M_r assumed for T-H-glycopeptide was 4800 and for leucoagglutinin 128 000. The desialylated erytrocytes (1.2%, v/v) were of group O

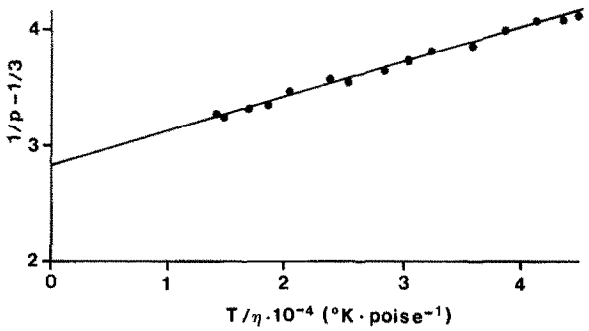

Fig.1. Perrin plot of the polarization of fluorescence of DNS-leucoagglutinin. The lectin was 5 μM in PBS. The equation for the straight line, calculated by the least-squares method was:

$$(\frac{1}{P} - \frac{1}{3}) = 2.81 + 0.29 \times 10^{-4}\ T/\eta$$

β (slope of the graph/intercept on the ordinate axis) was 10.32×10^{-6} and ρ_h was 105 ns at 25°C.

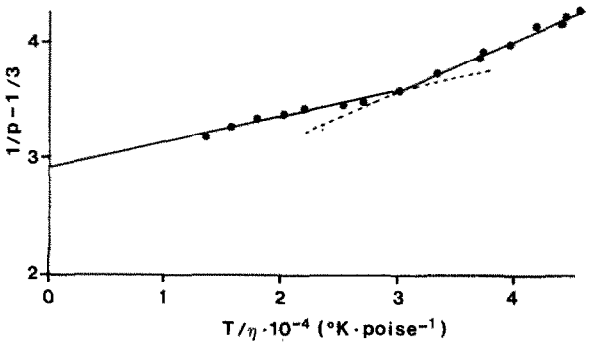

Fig.2. Perrin plot of the polarization of fluorescence of DNS-leucoagglutinin in the presence of T-H-glycopeptide. The lectin was 5 μM and T-H-glycopeptide 300 μM in PBS. The equation for the first linear slope (0–18°C) was:

$$(\frac{1}{P} - \frac{1}{3}) = 2.93 + 0.22 \times 10^{-4}\ T/\eta$$

β was 7.51×10^{-6} and ρ_h was 144 ns at 25°C. The equation for the second linear slope (24–37°C) was:

$$(\frac{1}{P} - \frac{1}{3}) = 2.27 + 0.41 \times 10^{-4}\ T/\eta$$

β was 18.06×10^{-6} and ρ_h was 60 ns at 25°C.

and in the presence of T-H-glycopeptide, respectively. The Perrin plot of dansylated leucoagglutinin calculated from a typical experiment in the absence of T-H-glycopeptide is shown in fig.1. The agreement with the Perrin linear low of depolarization is consistent with no conformational changes of the lectin due to the effect of temperature. From the ratio between the slope and the ordinate intercept, the rotational relaxation time (ρ_h) of the dansylated leucoagglutinin was calculated. A value of 105 ns was found. This value is very close to ρ_o of 121 ns calculated for a protein of 128 000 M_r with $\bar{V} = 0.73$ ml/g assumed for leucoagglutinin according to [14]. The value of fluorescence polarization of fluoresceinated leucoagglutinin, in the same temperature range, gave also a straight line relationship, but the ρ_h found (29 ns) was very low. We measured, with the same procedure, the ρ_h of fluoresceinated con A (Miles) and a value of 23 ns was found, which is also too low for a protein of M_r very close to that of leucoagglutinin. These results taken together indicate that the method of fluorescence polarization does not allow the determination of the true molecular volume of fluoresceinated lectins, probably because the τ of fluorescein is not adequate for proteins of M_r in the 10^5 range [12].

Fig.2 shows the Perrin plot of dansylated leucoagglutinin in the presence of T-H-glycopeptide. A deviation from the T/η axis with increasing the temperature is seen. From the polarization curve the initial and final linear slopes were calculated by the least squares method. From the two lines a value of ρ_h of 144 ns was calculated in the low temperature range (0–18°C), and a ρ_h of 60 ns in the higher temperature range (24–37°C). The increase of ρ_h of (leucoagglutinin)–(T-H-glycopeptide) in respect to that of free leucoagglutinin calculated from initial linear slope indicates an increment in the M_r of the rotating unit. This result may depend on the binding of 4 molecules of T-H-glycopeptide to the lectin. The half values of ρ_h obtained by the slope at higher temperatures indicates a temperature-dependent higher freedom of rotation of the protein when bound to the glycopeptide. A probable explanation may be a tetramer–dimer transition of the lectin in the higher range of temperature.

The Perrin plot of fluoresceinated leucoagglutinin bound to T-H-glycopeptide showed a similar deviation from linearity above 20°C and the ρ_h calculated from the data above this temperature resulted halved on respect to that obtained at lower temperatures (0–18°C: ρ_h = 28 ns; 22–37°C: ρ_h = 13 ns).

4. Discussion

Evidence has been reported [7–9] on the temperature-sensitive agglutinability of human erythrocytes by several lectins and the majority of them indicates stronger agglutination at 22°C and 37°C than at 4°C. Our results show that agglutination of human desialylated erythrocytes by leucoagglutinin is also temperature-dependent, but this lectin, like the naturally occuring ABO agglutinins, behaves as a 'cold agglutinin', inducing stronger agglutination at 4°C.

Different mechanisms were suggested to explain the temperature-dependent haemagglutinability by lectins:

(i) Structural rearrangement of the receptors on the membrane surface;
(ii) Modification of the number of the binding sites and/or their affinity for agglutinin;
(iii) Change in the quaternary structure of the lectin, such as tetramer–dimer transition of con A at low temperature [10].

The last modification is crucial because the agglutinating activity of con A is dependent on its tetravalency [15]. It seems unlikely that the first and the second mechanisms may be invocated to explain the higher agglutinability by leucoagglutinin at low temperatures. Previous evidence about con A proved that receptor-clusters formation [16] and affinity of binding sites for this lectin increase at higher temperature [17], and both changes positively affect agglutination.

By the method of fluorescence polarization, we found that leucoagglutinin, bound to specific glycidic receptors, such as T-H-glycopeptide, displays a freedom of rotation, with increasing temperature, higher than that predicted by linear Perrin low. For polimeric proteins this result is interpreted [18] as being due to dissociation into subunits of the protein, or to a relative independence of the subunits without true separation. In the latter condition the protein becomes more flexibile because of the weakening of the forces joining the subunits. One of these modifications seems to occur when leucoagglutinin bound to glycidic receptors reaches a temperature above 18°C. Such a conformational change of leucoagglutinin may explain the fall in its agglutinating power at 37°C.

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References

[1] Weber, T. H., Aro, H. and Nordman, C. T. (1972) Biochim. Biophys. Acta 263, 94–105.
[2] Miller, J. B., Hsu, R., Heinrikson, R. and Yachnin, S. (1975) Proc. Natl. Acad. Sci. USA 72, 1388–1391.
[3] Perlés, B., Flanagan, M. T., Auger, J. and Crumpton, M. J. (1977) Eur. J. Immunol. 7, 613–619.
[4] Serafini-Cessi, F., Franceschi, C. and Sperti, S. (1979) Biochem. J. 183, 381–388.
[5] Abbondanza, A., Franceschi, C., Licastro, F. and Serafini-Cessi, F. (1980) Biochem. J. 187, 525–528.
[6] Serafini-Cessi, F. (1980) FEBS Lett. 114, 299–301.
[7] Vlodavsky, I., Inbar, M. and Sachs, L. (1972) Biochim. Biophys. Acta 274, 364–369.
[8] Gordon, J. A. and Marquardt, M. D. (1974) Biochim. Biophys. Acta 332, 136–144.
[9] McKenzie, G. H., Sawyer, W. H. and Nicol, L. W. (1972) Biochim. Biophys. Acta 263, 283–293.
[10] Huet, Ch., Lonchampt, M., Huet, M. and Bernadac, A. (1974) Biochim. Biophys. Acta 363, 28–39.
[11] Montanaro, L. and Sperti, S. (1979) Methods Enzymol. 60, 712–719.
[12] Brand, L. and Witholt, B. (1967) Methods Enzymol. 11, 852.
[13] Goldstein, I. J., Reichert, C. M. and Misaky, A. (1974) Ann. NY Acad. Sci. 234, 283–295.
[14] Allen, L. W., Svenson, R. H. and Yachnin, S. (1969) Proc. Natl. Acad. Sci. USA 63, 334–341.
[15] Gunther, G. R., Wang, J. L., Yakara, I., Cunningham, B. A. and Edelman, G. M. (1973) Proc. Natl. Acad. Sci. USA 70, 1012–1016.
[16] Nicolson, G. L. (1972) Nature New Biol. 239, 193–197.
[17] Marciani, D. J. and Okazai, T. (1976) Biochim. Biophys. Acta 455, 849–864.
[18] Laurence, D. J. R. (1957) Methods Enzymol. 4, pp 200–201.