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Stabilization of insulin by alkylmaltosides. A. Spectroscopic evaluation

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Abstract

The aggregational behavior of native bovine insulin has been studied by circular dichroism and quasi elastic laser light scattering. The influence of a homologous series of alkylmaltosides on the self-association and aggregation of insulin was investigated. Significant stabilization of insulin was observed for the alkylmaltosides. Circular dichroism revealed that dodecylmaltoside was the most promising compound from a stabilizer stand point. Using dodecylmaltoside as the model compound, it was found that micelles were formed in the concentration range of the experiments and micell formation appeared to be important for the stabilization of insulin. Moreover, the results may suggest that insulin partitions into the micelles in an equimolar fashion and that insulin in this 'mixed micellar' form is relatively stable.

Keywords: Insulin; Peptide; Self-association; Aggregation; Laser light scattering; Alkylmaltosides

1. Introduction

Insulin is a polypeptide with a molecular weight of 5733 g/mole (bovine) consisting of 51 amino acids shared between two intramolecular chains. Insulin's conformational structure is well described (Marshall et al., 1971; Blundell et al., 1972; Pullen et al., 1976; Arquilla et al., 1978; De Meyts et al., 1978; Baker et al., 1988). The self-association and aggregational behavior of insulin has also been studied extensively because of the use of the drug in subcutaneous formulations for the treatment of diabetes (Brange, 1994).

The region on the insulin molecule responsible for aggregational behavior has been identified as a hydrophobic segment near the B-chain terminus (De Meyts et al., 1978; Baker et al., 1988). By exchanging amino acids in this border region, analogs with lower association tendencies were obtained (Brange et al., 1988). The association state of insulin at neutral pH in aqueous solution is predominantly hexamer (Hvidt, 1991). Removal of the region responsible for aggregation was

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shown to reduce association (Goldman and Carpenter, 1974). The fibrillation is a major problem for long-term storage and pump systems, and may affect the level of side effects encountered by diabetic patients in exogenous insulin treatment (Brange, 1987). It has been shown that aggregation of insulin in containers not only could be caused by denaturation at the solution/container interface but also by the constantly changing solution/air interface (Schrader and Pfeiffer, 1985). Moreover, as the temperature of a system is elevated, the aggregation increases dramatically.

Insulin association and aggregation has been studied by both circular dichroism and laser light scattering (Sato et al., 1983; Martindale et al., 1982; Bohidar and Geissler, 1984; Sluzky et al., 1991; Hvidt, 1991). Moreover, it is well known that micellar systems solubilize proteins. Insulin has been stabilized in such systems by many amphiphiles. Aggregation has most often been determined in an indirect manner as solution turbidity depression (Lougheed et al., 1983; Grau, 1985; Brange, 1987). Polypropylene glycol (PPG)/polyethylene glycol (PEG) block copolymer has been shown very effective in reducing the extent of aggregation (Thurow and Geisen, 1984; Chawla et al., 1985).

Alkylmaltosides were previously shown to stabilize insulin in solution against aggregation (Hovgaard, 1991; Hovgaard et al., 1992). The purpose of this present study was to verify the stabilization of insulin by dodecylmaltoside (DDM). Therefore, circular dichroism and quasi elastic laser light scattering was employed. Based on the characterization of DDM and DDM-insulin complexes, a model for stabilization has been proposed.

2. Materials and methods

2.1. Synthesis of alkylmaltosides

The synthesis of the alkylmaltosides has been described elsewhere (Hovgaard, 1991). Briefly, peracetylated bromomaltose was allowed to react with the fatty alcohols under anhydrous conditions. The protective acetyl groups were then hydrolyzed under alkaline conditions and the crude product was isolated. The products (octylmaltoside (OM), decylmaltoside (DM), dodecylmaltoside (DDM), tetradecylmaltoside (TDM), hexadecylmaltoside (HDM), and octadecylmaltoside (DDM) were isolated and purified by diethyl-ether precipitation followed by preparative flash-chromatography. The yields varied from 7 to 42% and the purities were above 99%, as determined by elemental analysis.

2.2. Insulin stability measured by circular dichroism

The stability of insulin in solution was studied under agitation. Bovine insulin (Sigma Chemical Co., St. Louis, MO) in a concentration of 0.1 mg/ml in 0.1 M phosphate buffered saline (PBS) of pH 8.0 was mixed with each of the maltosides in concentrations ranging from 0.2 mmole/ml (0.1 mg/ml) to 12.6 mmole/ml (6.4 mg/ml), equivalent to insulin: alkyl maltoside molar ratios of 1:11 to 1:720. Gentamycin (Sigma) was added in a concentration of 0.0025% to prevent bacterial growth. Agitation took place in a shaking water bath (37.0 $\pm$ 0.1°C) at a rate of 100 $\pm$ 1 strokes/min, 35 mm/stroke. Aggregation was followed by circular dichroic spectral analysis of insulin at 209 nm on a JASCO J-40A (Automatic Recording Circular Dichroism Spectropolarimeter, Japan) (Hovgaard et al., 1992).

2.3. Determination of critical micelle concentration (CMC)

CMC was determined by laser light scattering. Particle free borosilicate glass test tubes (7.5 x 100 mm) were used for all measurements. A series of concentrations for each maltoside was prepared in 0.1 M PBS of pH 8.0. The samples were filtered through a 0.2 μm Gelman Acrodisc syringe filter and sealed with a dust-free plastic stopper. The tube was placed in the sample holder of the laser light scattering goniometer (Brookhaven Instruments Corporation, Holtsville, N.Y.). The laser was equipped with a green Argon-ion laser with a power range of 10 mW–2 W and adjustable wavelength (4 880 and 5 145 Å). The laser appara-

tus has been described elsewhere (Cohen et al., 1990). The laser power was adjusted to 500 mW. All experiments were performed at a scattering angle of 90°. The scattering intensity and the size of micelles were measured as functions of concentration. All experiments were done in triplicate at room temperature (21°C).

2.4. Insulin stability measured by laser light scattering

Insulin was investigated in a concentration of 175 μM (1.0 mg/ml) in 0.1 M phosphate buffer of pH 8.0. Based on the CD results, DDM was chosen for this part of the study. Dodecylmaltoside was added in concentrations below and above CMC. The molar ratios of insulin to dodecylmaltoside studied were 1:0.6, 1:2.3, 1:11 and 1:45. Samples were prepared fresh less than 1 h prior to the start of experiments. The light scattering intensity of the solutions were measured daily. To study the pH dependent self-association, a series of 0.05 M phosphate buffers (pH 2.0–12.0) were prepared. Insulin was dissolved in the different buffers in a concentration of 1.0 mg/ml in order to minimize data collection time. Light scattering intensity and diffusion constants of these solutions were measured immediately.

2.5. Data analysis

CMC was determined from plots of scattering intensity versus concentration. The scattered light intensity is analyzed through an autocorrelation function (Cohen et al., 1990)

$$R(t) = \sum_t I_s(t) I_s(t + \tau) \quad (1)$$

where $I_s(t)$ is the intensity at a specific time and $I_s(t + \tau)$ at a later time, τ. In polydisperse systems, the autocorrelation function contains a sum of exponentials (Mazer, 1985)

$$R(t) = \left(\sum_i G_i e^{-D_i Q^2 \tau} \right)^2 \quad (2)$$

where G_i is the relative intensity scattered, D_i is, in the case of a polydisperse population, an average diffusional coefficient of the i^{th} particle and q is the size of the scattering vector. Assuming that the particles are spherical, the hydrodynamic radius (R_h) can be computed through the Stokes-Einstein equation (Mazer et al., 1976)

$$R_h = \frac{k_B T}{6\pi\eta D} \quad (3)$$

Insulin self-association can be studied by QELS with great advantages (Bohidar and Geissler, 1984; Hvidt, 1991; Sluzky et al., 1991). For molecular weight determinations, the ratio of scattered to incident light, corrected for geometric factors such as the detection angle of scattered light, is expressed by the Rayleigh ratio R_θ (Eisenberg and Crothers, 1979)

$$R_\theta = \frac{4\pi^2 n_o^2 \left(\frac{\partial n}{\partial c} \right)^2}{N_A \lambda^4} c M_w \quad (4)$$

where n_o is the solvent refractive index, N_A is Avogadro's number, M_w is the molecular weight, λ is the wavelength of the incident light and c the concentration of solute. Based on literature values for the concentration dependencies of refractive indexes for insulin and surfactant solutions, we have made the assumption that they can be equated (Mysels and Princen, 1959; Rosen, 1989; Hvidt, 1991). Based on the scattering data for insulin solutions, it is reasonable to assume that the predominant species of insulin in neutral solutions is the hexamer (Brange, 1994). A close examination of equation 4 then reveals the following simple relationship

$$R_\theta = K_c M_w \quad (5)$$

in which K is a constant representing the bracketed expression in equation 4. From equation 5, the molecular weight of the scattering species can then easily be estimated.

3. Results and discussion

3.1. Insulin stability measured by circular dichroism

At pH 8.0 the circular dichroic spectrum of insulin possesses a significant negative trough at

220 nm and 209 nm, corresponding to insulin's specific tertiary structure. As insulin aggregates, this structure gradually changes. Since the negative trough at 209 nm has the strongest spectral activity, this wavelength was followed. In this experimental series, the molar ellipticity was found to range from -7.5×10^3 to -8.5×10^3 deg cm^2/dmole. This was not changed by the presence of any of the stabilizers. The first sign of aggregation could be registered as changes in the CD spectrum, followed by visual opaqueness. The results are given in Fig. 1. The highest stabilization was observed for DM (1:64 (w/w)) and DDM (1:4 (w/w)). After 57 d, the DDM-insulin complex was found to be stable and to possess full biological activity after i.v. injection in rats as determined by blood glucose levels. These results suggest that the effect of the alkylmaltosides is governed by their length and complex ratio to insulin. Moreover, the stabilization was found to correlate with the CMC. This will be touched upon later.

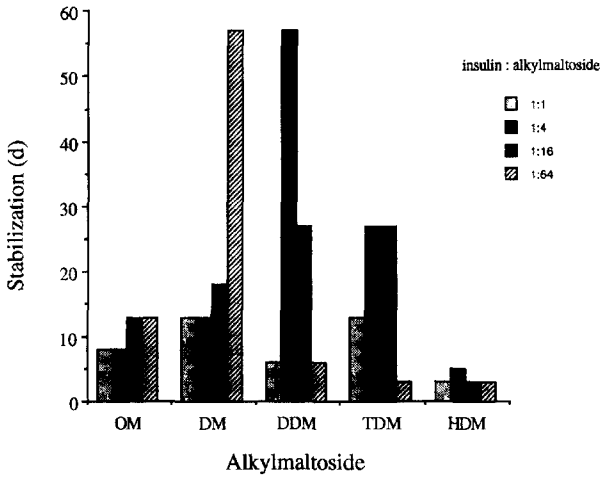

Fig. 1. Time of insulin stabilization by the stabilizers; OM, DM, DDM, TDM, HDM and ODM in the w/w ratios 1:1, 1:4, 1:16 and 1:64 (insulin to stabilizer) equivalent to molar ratios of 1:11, 1:45, 1:180 and 1:720 in the case of DDM. Time of insulin stabilization is determined to be at the time of 90% preservation of initial ellipticity (day 1) in the circular dichroic spectrum of insulin.

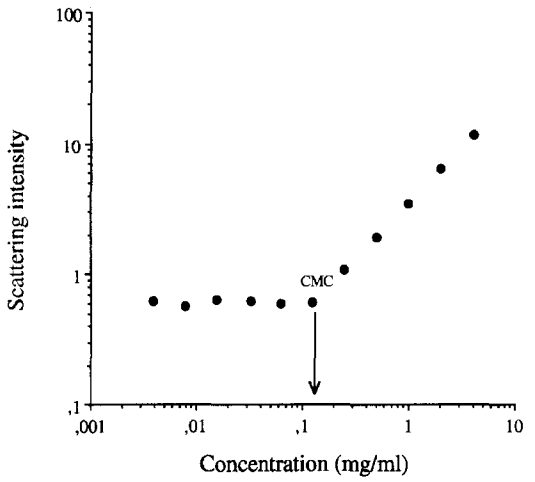

Fig. 2. Laser light scattering intensity as a function of concentration of dodecylmaltoside. The critical micelle concentration (CMC) is observed at the break on the curve.

3.2. Determination of critical micelle concentration

The critical micelle concentration was found at the point of discontinuity in the plots of scattering intensity versus concentration, as shown for dodecylmaltoside in Fig. 2. The plot allows for a precise determination of the event. All of the alkylmaltosides showed micelle formation, with Table 1 listing the CMC values. The shorter chain maltosides (octyl to dodecyl) show CMC of 5–0.24 mM, along with a micellar size of about 33 Å. This correlates with values reported for alkylglucosides by Rosen (1989). It was reported that octyl- to dodecyl- glucoside, which have smaller polar head groups, but the same alkyl chain

Table 1
Critical micelle concentration of alkylmaltosides

Compound	Critical micelle concentration	
	[mg/ml]	[mM]
Octylmaltoside	2.3	5.07
Decylmaltoside	0.80	1.66
Dodecylmaltoside	0.12	0.24
Tetradecylmaltoside	0.075	0.13
Hexadecylmaltoside	0.0081	0.014
Octadecylmaltoside	0.0017	0.0029

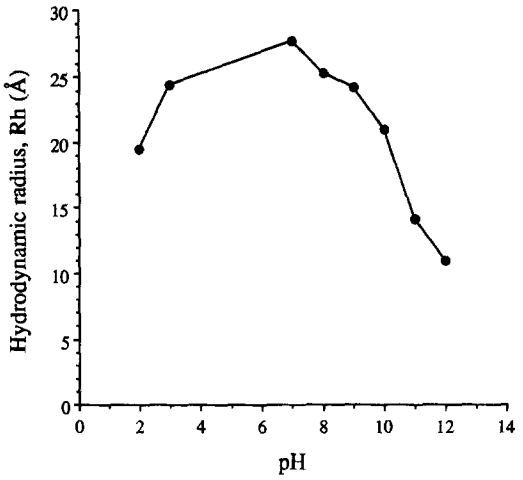

Fig. 3. Insulin aggregational pattern over a pH range from 2 to 12. Hydrodynamic radii of insulin as a function of pH in the solution measured by dynamic laser light scattering.

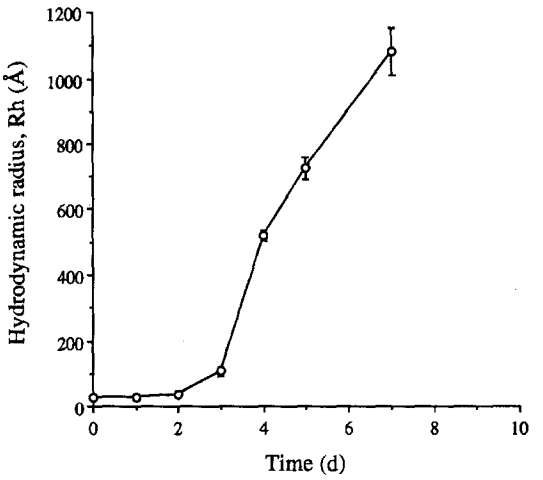

Fig. 4. Hydrodynamic radius of insulin, 1 mg/ml in 0.1 M PBS (pH 8.0), as a function of time measured by dynamic laser light scattering.

lengths, had CMCs from 25 mM to 0.19 mM (Rosen, 1989). Moreover, the hydrodynamic radii for micelles are in the range of 25–150 Å for an ionic amphiphile compound like sodium dodecyl sulfate (Mazer, 1985). The solubility of the maltosides up to tetradecylmaltoside was not reached within the concentration range of the experiment. However, longer chain analogs were less soluble and showed some turbidity at the high concentrations. The sizes of the micelles increased slightly with increasing length of the alkyl chain. An initial 'plateau' at a size of 33 Å for OM, DM and DDM could be caused by the bulky nature of the maltose head group on the shorter chain alcohols. The micellar sizes of the largest maltosides were not determined.

3.3. Insulin stability measured by laser light scattering

The association state of insulin was determined at pH from 2 to 12. The insulin solutions showed predominantly dimers at low pH and monomer at high pH, with radii of 19 and 11 Å, respectively (Fig. 3). These results are in full agreement with observations by Bohidar and Geissler (1984), who found R_h for monomer insulin to be 12–13 Å. At neutral pH, the associational state was hexamer, showing a size of 28 Å which was in accordance with other reports (Brange, 1994). The aggregation was followed in neutral solutions of insulin. Fig. 4 shows the change in the hydrodynamic radius with time. The radius increased from 27 Å at time zero to over 1000 Å after just 1 week. The increase in the hydrodynamic radius suggests macromolecular aggregation. When dodecylmaltoside was added, insulin did not aggregate as readily (Fig. 5). The hydrodynamic radius is plot-

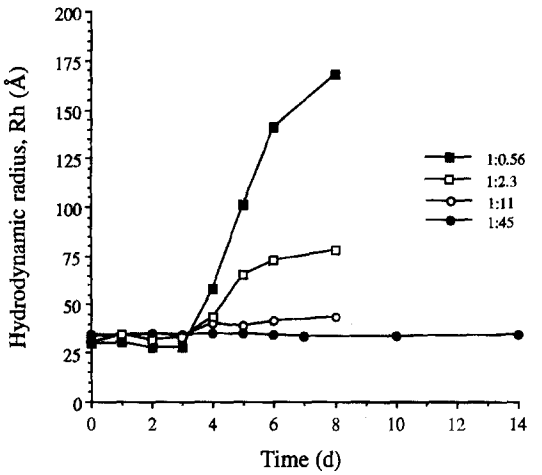

Fig. 5. Hydrodynamic radius of insulin, 1 mg/ml in 0.1 M PBS (pH 8.0), containing dodecylmaltoside in various molar ratios, as a function of time measured by dynamic laser light scattering.

Table 2
QELS data for pure insulin and DDM solutions

	R_h (Å)	I_{ins}/I_t	C (mg/ml)
Insulin	28	0.7	1
Dodecylmaltoside	35	3.2	4

ted as a function of time for all the tested concentrations of dodecylmaltoside. Samples containing insulin and maltosides showed an initial hydrodynamic radii ranging from 27 to 33 Å, depending on the concentration of dodecylmaltoside. The solutions tested ranged from 98 x 10^{-3} to 7.8 mM dodecylmaltoside. As the concentrations of dodecylmaltoside were raised, higher stabilization was observed.

The theoretical description of the insulin dodecylmaltoside complex composition is not easy. In this study, we will try to discuss the possible ways of stabilization. QELS measurements on the pure components gave the values given in Table 2. Insulin in a concentration of 1 mg/ml can be assumed to represent insulin hexamer solution characteristics. Therefore, the molecular weight of the scattering species is 34 400 g/mole. Equation 6 states that toluene normalized scattering intensity of a mixture of solutes with different molecular weights is proportional to the sum of the product of concentration, c, and molecular weight, M (Mazer et al., 1976)

$$\frac{I_i}{I_t} = K\sum_i c_i M_i \quad (6)$$

If equation 6 is used on the pure insulin solution, the constant K can be determined.

$$\frac{I_{ins}}{I_t} = K_c M_w \Rightarrow K = \frac{0.7}{1 \cdot 34400} = 2 \times 10^{-5}$$

K can be used as a good approximation for the calculation of the molecular weight of a micelle. From the results for pure dodecylmaltoside solution

$$M_w = \frac{3.3}{4 \cdot 2 \times 10^{-5}} = 41000$$

This suggests that the aggregation number of DDM in a micelle is approximately 80. Based on this knowledge, Table 3 has been constructed. The stabilization measured by CD under agitation and QELS without agitation suggests that DDM is stabilizing insulin in solution. In the case of agitation, an optimum stabilization was reached when 0.6–2.3 mole of micelles were present per mole of insulin hexamer. This indicates that an interaction of the insulin molecule with micelles of DDM is essential for the stabilization. However, if 9 moles of DDM micelles were present, destabilization was observed. This could be due to denaturation of the insulin molecule. The maximum stabilizing concentration of micelles was confirmed by QELS where the initial hydrodynamic radius of the mixture of insulin and dodecylmaltoside micelles was preserved for at least 2 weeks.

In conclusion, alkylmaltosides were shown to have a great potential as stabilizers for insulin. The concentration range for stabilization determined by circular dichroism correlated with the CMC values for DM and DDM. It was not possible to show this for other maltosides. DDM may stabilize insulin in a mixed micellar fashion. At this point, only speculation can be made on how the stabilization of insulin by DDM micelles takes place. The micelles may pose as an attractive phase for the insulin dimer or monomer. Therefore, these species can advantageously partition into the micelles instead of taking part in aggregation of insulin in higher states. This will have to be studied in the future. However, if this hypothesis is true, the reduction in molecular weight of the insulin form presented to the mucosal surface can be reduced and a higher transport expected.

Table 3
Various molar ratios of insulin to DDM calculated on basis of QELS

Insulin:DDM (w/w)	Insulin:DDM (mol/mol)	Insulin:micelles (mol/mol)
1:0.05	1:0.56	1:0.007
1:0.2	1:2.3	1:0.028
1:1	1:11	1:0.14
1:4	1:45	1:0.6
1:16	1:180	1:2.3
1:64	1:720	1:9

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