Relationship between isoelectric point of native and chemically modified insulin and liposomal fusion

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Native insulin causes fusion of negatively charged liposomes in the pH range from 3.0 to 5.5. In marked contrast, insulin with all three amino groups succinylated did not show fusion ability at any pH. On the other hand, insulin amidated with glycine methyl ester with all six carboxyl groups blocked shifted its activity to higher pH, showing a pH range of activity from 3.0 to 7.4. When the carboxyl groups were recovered by hydrolysis of methoxyl groups from glycine methyl ester-treated insulin, the protein obtained (glycyl-insulin with six free carboxyl groups) behaved as native insulin. A good correlation between the isoelectric point values of insulin and its derivatives and their fusion properties was found.

INTRODUCTION

Despite the importance of membrane fusion in a wide variety of biological processes, little is known about the molecular events accompanying fusion or the way in which cells control fusion of their membranes [1]. Fusion does not occur indiscriminately; it must be a precisely regulated process with regard to time, extent, cellular location and stimulus specificity.

The potential role of proteins in natural membrane fusion has repeatedly been suggested [2]. Due to the complexity of biological membranes, the fusion process was mainly studied in membrane model systems, more amenable to experimental analysis, such as liposomes. In this latter system, peptides and proteins were shown to be efficient fusion inducers and were postulated as ‘fusogenic agents’ of biological relevance [3–16]. Also, the molecular mechanism of liposome fusion is still poorly understood. Fusion of liposomes modulated by proteins can be generalized as either Ca^{2+} -dependent or Ca^{2+} -independent (see [17] for a review). Recently our laboratory has demonstrated the fusogenic ability of several proteins in the absence of Ca^{2+} [12–15]. One of these, insulin, can induce fusion of negatively charged small unilamellar vesicles at low pH, but not at neutral pH [13,14]. Four different procedures were used in support of this conclusion: resonance energy transfer, light scattering, Sepharose chromatography and electron microscopy [13,14]. This fusion process was inhibited by high salt concentration. Uncharged vesicles did not interact with insulin at any pH. These facts suggested the predominance of electrostatic interactions during the liposomal fusion. It was proposed that such interaction induces a destabilization of the bilayer, which becomes susceptible to fusion. Changes in biological membrane fluidity by insulin have been reviewed elsewhere [18]. It may be that insulin can promote membrane fusion following its internalization by receptor-mediated endocytosis, once it becomes exposed to the acidic environment that promotes ligand–receptor dissociation inside the intermediate endosome compartment [13,14]. Con-

sidering the physiological implications of this hypothesis, it was interesting to investigate the effect of variations in pH on insulin-mediated fusion.

In this paper we indicate that the isoelectric point (pI) of insulin governs the limits of its fusion ability by changing the pH-dependence of insulin-mediated fusion by chemical modification of the protein and consequently its isoelectric point.

MATERIALS AND METHODS

Phosphatidylcholine (PC) and phosphatidic acid (PA) from egg yolk, and small unilamellar phospholipid vesicles of 20–30 nm, were prepared as previously described [12]. The purity of phospholipids was checked by t.l.c. on silica gel H, with chloroform/methanol/ NH_4OH (12:7:1, by vol.) as solvent. Lipid mixing between vesicles was monitored by resonance energy transfer using cholesteryl anthracene-9-carboxylate (CA9C) (donor) and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (NBD-PE) (acceptor) as described by Morero *et al.* [12]. The mixture (2 ml final volume), contained 20 nmol of phospholipids (PC/PA, 4:1), 0.6 nmol of CA9C and 1.8 nmol of NBD-PE. The fluorescent CA9C and NBD-PE were incorporated in different vesicle populations. Equal volumes of CA9C vesicles and NBD-PE vesicles were mixed in 10 mM-phosphate/citrate buffer at different pH values as indicated in the figures. The emission spectra at 470 nm of fusion mixtures were obtained 1 min after the addition of insulin or its derivatives by excitation at 360 nm. The efficiency of resonance energy transfer is defined by the equation:

$$\text{RET} = 1 - F/F_0$$

where F_0 is the fluorescence emission intensity of CA9C in the absence of fusogenic agents and F the fluorescence in the presence of the protein to be tested.

Amidation of carboxyl groups of insulin with glycine methyl ester was carried out according to the procedure described by Hoare & Koshland [19] under experimental

Abbreviations used: CA9C, cholesteryl anthracene-9-carboxylate; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; PC, phosphatidylcholine; PA, phosphatidic acid.

Fig. 1. Preparation of insulin derivatives

A, succinyl-insulin; B, methyl ester glycyl-insulin; C, glycyl-insulin.

conditions in which the six carboxyl groups of insulin were blocked. The insulin derivative was separated from the reagent mixture by gel filtration on a Sephadex G-25 column (0.9 cm $\times$ 20 cm) equilibrated with 1.0 M-acetic acid. A second Sephadex G-25 column equilibrated with 20 mM-Tris/HCl buffer, pH 7.2, was used to separate acetic acid. The protein solution was concentrated by evaporation under reduced pressure, the pH was adjusted to 7.6 and kept at 4 °C for 3 h. The insoluble methyl ester glycylated-insulin was obtained by centrifugation at 3000 rev./min for 5 min.

Succinylation of amino groups of insulin was performed as indicated by Klapper & Klotz [20]. Subsequently, the solution was passed through a Sephadex G-25 column equilibrated with 1.0 M-acetic acid. Under these conditions the unmodified insulin was eluted in the void volume, whereas the succinyl-insulin derivative was included in the column. The fraction containing the succinyl-insulin was then chromatographed on a Sephadex G-25 column equilibrated with 20 mM-Tris/HCl buffer, pH 7.4. In this column the succinyl-insulin was eluted in the void volume. After concentrating the protein solution, the pH was adjusted to 4.3 and the solution was kept at 4 °C for 3 h. The insoluble protein was sedimented by centrifugation at 3000 rev./min for 5 min. Free amino groups were not detectable in the product with the fluorescamine assay.

The hydrolysis of methoxy group from methyl ester glycyl-insulin was carried out by overnight incubation at 4 °C in 7 mM-NaOH as described in [21]. The protein was purified by isoionic precipitation at pH 5.5. All the proteins derivatives were free of unmodified insulin, as checked by h.p.l.c. The isoelectric points of insulin and its derivatives were estimated by isoionic precipitation and by a method based upon the pH-dependent binding affinity of amphoteric molecules developed by Yang & Langer [22].

RESULTS AND DISCUSSION

Fig. 1 shows the amino and carboxyl groups of bovine insulin that were modified in order to obtain insulin derivatives with different isoelectric points. After the alkaline hydrolysis of methyl ester glycyl-insulin an

Fig. 2. Effect of pH on the fusion ability of 5×10^{-6} M-insulin and its derivatives

Insulin (●), succinyl-insulin (○), methyl ester glycyl-insulin (△), and glycyl-insulin (□).

insulin derivative molecule with six free carboxyl groups (as the original insulin) was obtained.

The fusion abilities of insulin derivatives as a function of pH are plotted in Fig. 2. In a previous paper [13] we demonstrated that the optimal pH for native insulin, with pI of 5.6, was in the range of 3.6 to 3.9. As can be seen in Fig. 2, with increasing pH the extent of fusion decreased, reaching a minimum at pH 5.5 and being zero at pH 6.5. Methyl ester glycyl-insulin derivative, with pI of 7.4, is active as a fusogenic agent at pH 6.5, reaching a minimum activity at pH 7.4–7.6. When this insulin derivative was demethoxylated and six carboxyl groups of the protein were recovered, the new derivative (glycyl-insulin) displayed a pH dependence similar to that of the original insulin. The succinyl-insulin derivative, with pI of 3.8–4.2, did not show fusion ability at any pH assayed. It was previously shown that blocking of the three available amino groups with fluorescamine did not inhibit the binding of insulin to negatively charged vesicles [14]. This fact ruled out the possibility that blocking of amino groups caused the inhibition observed with the succinyl-insulin derivative. It is the replacement of the amino groups by carboxyl groups and the subsequent pI change that appears to be the reason for the observed effect. Fig. 3 shows the dose-response curves of insulin and its derivatives at pH 4.0 and 5.5. Neither insulin nor its derivatives showed fusion activity with uncharged vesicles at any pH (not shown).

It is useful to compare the similarities of the insulin-induced fusion with other well-studied systems. At pH < pI, insulin is a polycation. Fusion of negatively charged vesicles induced by polycations has been thoroughly studied with polylysine [23–26] and polyhistidine [8]. In the case of polyhistidine, the fusion activity is only observed at pH < pI [8]. The fusogenic activity of polyhistidine could be correlated with the level of positive charge of the polymer at a given pH.

Insulin-induced fusion of vesicles (PC/PA, 4:1) is

Fig. 3. Insulin and its derivatives induced fusion at pH 4.0 (a) and pH 5.5 (b)

Insulin (●), succinyl-insulin (○) and methyl ester glycy-insulin (△).

critically dependent upon the pH [13]. The degree of dissociation determined by acid-base titration curves of phosphatidic acids gave pK_1 values in the range from 2.8 to 3.9 and pK_2 values in the range from 8.0 to 9.0 [27,28]. At the pH range from pK_1 to pK_2 at least one net negative charge is present in PA [27]. Below pH 2.5 the negative charges of PA are neutralized. Insulin has three positive charges at pH 4.0 and ten at pH 2.6 [29]. Electrostatic interaction between insulin and negatively charged vesicles is only possible in the pH range from 3.0 to 5.5, but not at pH values lower than 3.0 or higher than 5.5 [13]. The interaction at low pH is limited by the negative charge on the vesicles whereas the interaction at high pH is limited by the positive charges of the native and chemically modified insulin (Fig. 2).

The experimental results presented here are in agreement with the previous hypothesis [13,14] that electrostatic interactions between negatively charged vesicles and insulin could be the most important early event in the process of protein-induced membrane fusion. Moreover, they indicate that the pI governs the limits of fusion ability of insulin and its derivatives, as shown by the right-hand profile of the pH curves (Fig. 2). Obviously, the pH-activity profile of each protein was independent of the degree of ionization of the phospholipid head groups in the vesicles, since this parameter remained unchanged under the experimental conditions used.

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