Communication

A Preformed, Ordered Structure of a 25-residue Peptide Derived from a Major Histocompatibility Complex Class I Antigen Is Required to Affect Insulin Receptor Function*

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Jan Stagsted†, Walter A. Baase§, Avram Goldstein‡, and Lennart Olsson‡

From †Receptron, Inc., Concord, California 94520, the §Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403, and the ‡Department of Pharmacology, Stanford University School of Medicine, Stanford, California 94305

It was recently shown that a 25-residue peptide, Dκ-(61-85), derived from the α1 domain of a murine major histocompatibility class I molecule (H-2Dκ), affects insulin receptor functions (Hansen, T., Stagsted, J., Pedersen, L., Roth, R. A., Goldstein, A., and Olsson, L. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3123–3126; Stagsted, J., Reaven, G. M., Hansen, T., Goldstein, A., and Olsson, L. (1990) Cell 62, 297–307). We now report that this peptide can reversibly assume a biologically active or inactive state as measured in the rat adipocyte glucose uptake assay, implying that the peptide has at least two interconvertible conformations. The peptide has an ordered conformation in 0.1 M HCl or 0.1 M NaCl stock solution as shown by circular dichroism, but has a disordered molecular structure and is inactive when dissolved in H2O. The biologically active peptide forms liquid crystals at the stock solution concentration (1 mM), so the CD spectra do not provide information on the secondary structure. Under all conditions tested, biological activity (measured after transfer to assay buffer) is associated with an ordered conformation in stock solution. Biological activity and an ordered conformation of the peptide in H2O stock solution can be induced by increasing ionic strength (>100 mM NaCl for maximal effect) or increasing pH (>5 for maximal effect). The induction rate of the ordered conformation is slow with a half-maximal value obtained after ~20 min. Both biological activity and the ordered structure are lost upon heating of stock solution to 90 °C or upon transfer to assay buffer. A similar correlation of ordered structure with biological activity was observed with two truncated peptides derived from Dκ-(61-85). It is inferred from these results that the Dκ-(61-85) peptide and related peptides only affect insulin-stimulated glucose uptake in rat adipocytes if they have assumed an ordered conformation in stock solution prior to transfer to assay buffer and exposure to cells.

Biological activity of some peptides can be potentiated by amino acid modifications that enhance their amphiphility and that often also results in an α-helical secondary structure (9–5). In line with this, a correlation between biological activity of peptides and their solvent conditions has been demonstrated (6, 7). We recently reported that a 25-residue peptide, Dκ-(61-85), derived from the α1 domain of a murine major histocompatibility complex (MHC) class I antigen, affects key insulin receptor functions, including tyrosine kinase activity, insulin-stimulated glucose uptake, and insulin-induced receptor internalization (1, 2). We now report that the peptide, which has an amino acid sequence compatible with an amphiphilic secondary structure, can occur both in a biologically active and in an inactive state. The active form can be reversed to an inactive form by changing the solvent of the peptide stock solution from HCl or NaCl to H2O. Circular dichroism studies show that the active peptide in NaCl stock solution has an ordered, complex structure, whereas the inactive peptide in H2O has a spectrum consistent with a disordered structure. Further, and as a novel observation, the ordered conformation of this amphiphilic peptide must be assumed in the stock solution prior to transfer to assay buffer and exposure to cells. This peptide differs from peptides with membrane affinity, which only assume a secondary structure upon interaction with cell membranes (3). It is concluded that the Dκ-(61-85) peptide may assume different, but interconvertible conformations, that differ in biological activity, and that the biological potency might be enhanced by modifications that stabilize the active conformation(s).

MATERIALS AND METHODS

Peptides—The sequence of the peptide designated Dκ-(61-85) was derived from the α1 domain of the heavy chain of murine MHC class I (H-2Dκ). Other peptides used in this study are Dκ-(61-84) and Dκ-(69-85). The sequence of Dκ-(61-85) is shown in Fig. 1. The biological activity of the peptide was described previously (1, 2). All peptides were synthesized by Applied Biosystems, Inc. (Foster City, CA). The crude peptides were purified by preparative HPLC. Each peptide was more than 95% pure, as judged by analytical HPLC monitoring of absorbance at 214 and 278 nm (Fig. 1). Identities of the peptides were confirmed by amino acid composition and mass spectrometry.

After purification, peptide was lyophilized overnight from CH3CN/trifluoroacetic acid and then solubilized at 25 °C in one of two stock solvents at 1 mM. HCl (Pierce Chemical Co., Sequanal grade) or H2O (Baxter, Burdick & Jackson Division, HPLC grade), as illustrated in Fig. 1 (step 1). Sodium chloride (final concentration, 0.1 M) was added in some experiments to the stock solution of water-solubilized peptide (Fig. 1, step 2). In step 3 (incubation in assay buffer without cells), incubation time was varied from 0 to 180 min. The biological assay (Fig. 1, step 4) was in Krebs-Ringer/HEPES buffer (KRH; 80 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 50 mM HEPES, pH 7.2) supplemented with 5% bovine serum albumin (KRHB). Irrespective of the solvent history of the peptide stock solutions, the final assay conditions were always identical.

Glucose Uptake by Adipocytes—Adipocytes were prepared as described (2). Briefly, minced epididymal fat pads were incubated in KRHB with 5 mM d-glucose and 1 mg/ml collagenase (type I, Worthington) and digested for 1 h at 37 °C. The adipocytes were washed five times (the conventional three washes result in substantial peptide degradation in KRHB), with each wash performed in 10 times the

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† The abbreviations used are: MHC, major histocompatibility complex; KRH, Krebs-Ringer/HEPES; KRHB, KRH/bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high pressure liquid chromatography.
Peptide Conformations and Biological Activity

**RESULTS**

**Effect of Solvents on Peptide Activity**—Fig. 2A shows that, when diluted in KRHB to a final concentration of 30 μM, the peptide from the stock solution in H2O had no effect on the insulin-stimulated glucose uptake by rat adipocytes, whereas peptide dissolved in 0.1 M HCl in the stock solution enhanced insulin-stimulated glucose uptake. The difference between biologically active and inactive peptide was significant (p < 0.001, t test). Further, the stock solution effects on biological activity of the peptide were fully reversible as demonstrated by the activity of H2O- or HCl-solubilized peptides upon lyophilization and subsequent resolubilization in HCl or H2O (Fig. 2A). It was later found that the biological activity could be induced in the inactive peptide from the H2O stock solution by addition of NaCl to a final concentration of 0.1 M at least 0.5 h prior to transfer to assay buffer. Therefore, biologically active peptide was, for most of the following experiments, obtained from a H2O-solubilized peptide stock solution to which NaCl was added (NaCl stock solution). Fig. 2B shows that the biological activity of the peptide from NaCl stock solution had an EC50 of 12 μM and that the peptide from H2O stock solution was about 20-fold less potent. Peptide concentrations higher than 300 μM could not be tested, as precipitation became significant, and the exact difference in biological potency between peptide in NaCl and H2O stock solutions was therefore not possible to estimate.

HPLC analysis on a reversed-phase C18 column with a gradient of 20-40% CH3CN in 5 mM trifluoroacetic acid over 5 min gave almost identical retention times (mean ± S.E., n = 4) for stock solution of the peptide in HCl (2.96 ± 0.03 min) and H2O (3.00 ± 0.02 min), with no indication of twin peaks in several coelution runs (data not shown). HPLC analysis of supernatants from adipocytes incubated with 3H-labeled peptide at 30 μM for 30 min in assay buffer showed ~20% degradation (data not shown), irrespective of whether the stock solution of 3H-labeled peptide had been prepared in HCl or H2O.

**Circular Dichroism Analysis of the Peptide and Truncated Derivatives**—The CD spectrum for the peptide in H2O indicates a random coil conformation, whereas the spectrum for the peptide in NaCl implies a complex, ordered structure (Fig. 3). The CD spectrum for the peptide in NaCl was independent of the distance between cuvette and detector, thus excluding chiral scattering artifacts (data not shown). The CD analysis of two truncated derivatives of the peptide, D(61-85) and

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**Fig. 1.** Upper left, amino acid sequence of D(61-85). Lower left, HPLC profiles of D(61-85), D(69-85), and D(61-84) on reversed-phase chromatography on an analytical C18 column eluted with a gradient of CH3CN (5-40% over 5 min) in 5 mM trifluoroacetic acid. The purity of each peptide was >95% as judged by absorbance at 214 nm. Right, flow diagram for peptide application in the glucose uptake and CD assays (see also "Materials and Methods").
Peptide Conformations and Biological Activity

D\text-superscript-\text-superscript-k-(61-84), demonstrated, for D\text-superscript-\text-superscript-k-(69-85) (biologically active), a pattern similar to the peptide in NaCl and, for D\text-superscript-\text-superscript-k-(61-84) (biologically inactive), a random coil spectrum, although shifted to the right. The inset shows the rate at which the CD signal at 220 nm changes from a random coil spectrum to a spectrum for a complex ordered structure after addition of NaCl to the peptide stock solution in H\text-superscript-\text-superscript-2O.

Peptide stock solution (0.9 mM) in NaCl was found to be birefringent with characteristics of liquid crystals when observed between crossed Polaroid filters, whereas peptide stock solution in H\text-superscript-\text-superscript-2O was nonbirefringent.

**Birefringence Analysis**

**Concentration of NaCl**

![Concentration of NaCl Graph](image)

**pH**

![pH Graph](image)

**Temperature**

![Temperature Graph](image)

**Time**

![Time Graph](image)

**Glucose Uptake**

![Glucose Uptake Graph](image)

**DISCUSSION**

Previous studies have shown that the peptide affects key insulin receptor functions, including tyrosine kinase activity, insulin-stimulated glucose uptake, and insulin-induced receptor internalization (1, 2). In the present study, biological activity was determined with the rat adipocyte glucose uptake assay. The biological activity of the peptide from NaCl stock solution was compared to that from H\text-superscript-\text-superscript-2O stock solution. The peptide from NaCl stock solution was incubated at 25 °C for 90 min and then incubated at 37 °C for 30 to 90 min in the indicated concentration of NaCl. The peptide was then diluted in assay buffer with insulin and added to the adipocytes at 30 μM for the standard 60-min glucose uptake assay. For CD measurements, the peptide was diluted to 30 μM in KRH, and the CD signal at 220 nm was recorded within 1 min after dilution.

Each point for both biological activity (open symbols) and CD signal (filled symbols) represents mean ± S.E. of three experiments, where maximal biological or CD signal was set to 100% in each experiment. The rate of the CD signal change at 220 nm after addition of NaCl to 0.9 mM D\text-superscript-\text-superscript-k-(61-85) stock solution in H\text-superscript-\text-superscript-2O is shown in the inset.

The biological activity and CD measurements were performed as in A. The data points (mean of triplicate samples) for both biological activity and CD signal are from three separate experiments, where maximal biological or CD signal was set to 100% in each experiment. The highest ionic strength (40 mM final) contributed by the added base or acid was insufficient to induce activity by ionic strength alone (see A). D\text-superscript-\text-superscript-k-(61-85) in NaCl at a concentration of 0.9 mM was heated from 30 to 90 °C, and samples were taken out at various temperatures, transferred to 37 °C assay buffer, and tested in the 60-min glucose uptake assay. The results are means ± S.E. of four experiments, where the CD signal at 30 °C is set to 100%. D\text-superscript-k-(61-85) in H\text-superscript-\text-superscript-2O at a concentration of 0.9 mM was heated to 90 °C and samples were taken out at various temperatures, transferred to 37 °C assay buffer, and tested in the 60-min glucose uptake assay. The results are means ± S.E. of four experiments, where the CD signal at 30 °C is set to 100%.

Most of the peptide (80 ± 1%, n = 3) in NaCl stock solution could be pelleted by ultracentrifugation at 100,000 × g for 60 min, compared with only 10 ± 3% (n = 3) of the peptide in H\text-superscript-\text-superscript-2O stock solution. The biological activity of the solubilized peptide in NaCl stock solution could not be detected in the supernatant after ultracentrifugation, implying an association between the biologically active peptide and large peptide aggregates.
(69–85) dissolved in H_2O has a CD spectrum comparable with D^+(61–85) in NaCl, whereas the spectrum for the stock solution of the inactive D^+(61–84) in HCl is comparable with D^+(61–85) in H_2O. The 8 amino-terminal residues, Glu-Arg-Glu-Thr-Gln-Ile-Ala-Lys, in the peptide are thus prohibitive for generation of ordered conformation in H_2O. Similarly, the data for D^+(61–84) show that the absence of the carboxy-terminal tyrosine 85 impairs formation of ordered structure of the peptide in 0.1 M HCl.

A typical α-helical CD spectrum shows local minima at 222 and 206 nm (8), but the spectrum for the peptide in NaCl has local minima that are both shifted leftward ~5–10 nm. This suggests a complex structure that may contain both α-helix and β-sheet conformations. However, under these conditions the biologically active peptide forms liquid crystals, and the CD spectra can therefore not be used to deduce any information on the secondary structure of the individual peptide molecules. The slow rate of induction (minutes) of the ordered structure after addition of NaCl to the peptide in H_2O suggests that the biologically active conformation requires interaction between peptide molecules and is consistent with the formation of liquid crystals. In contrast, intramolecular conformational changes are known to occur in milliseconds.

The linear sequence is not sufficient for the biological effect of the peptide. The peptide must not only acquire a certain conformation in order to be biologically active, but it is also evident that this active conformation must exist in the stock solution prior to addition to the assay buffer. Both the complex, ordered structure and biological activity disappear if the active peptide is incubated for 20–30 min in cell-free KRH prior to the glucose uptake assay.

Peptide sequence alterations that result in increased amphiphilicity and α-helix formation also often result in enhanced biological activity of several hormones such as calcitonin (9–11) and β-endorphin (12–14). In addition, the assumed association between amphiphilic α-helix formation and biological activity has been explored successfully to design biologically active peptide analogues of the honey bee venom biologically active peptide has been explored successfully to design monomeric or oligomeric peptides. It may also be noted that small differences in the linear sequence of peptides, which affect their amphiphilicity, can have a major impact on their binding to MHC class I and class II antigens and, thereby, their immunogenicity (16, 17). Our observations described above indicate, however, that conformational differences of a peptide caused by different solvent conditions may result in variable immunogenicity, which therefore is not solely determined by the amino acid sequence.

Enhancement of the potency of active peptide is desirable for several reasons, including the fact that low potency has thus far excluded binding studies to determine binding site(s) and affinities to insulin receptor and whole cells. Based on the observations above, we suggest that enhancement of potency may be achieved by peptide modifications that promote and stabilize the ordered conformation associated with biological activity.

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| Table 1 Correlation between biological activity and CD signal |
|-------------------------------------------------------------|
| Biological activity | Ordered CD signal |
|---------------------|-------------------|
| D^+(61–85) in H_2O  | –                 |
| D^+(61–85) in NaCl   | +                 |
| Increasing pH^*     | +                 |
| Increasing ionic strength^* | +             |
| Melting^*           | –                 |
| Decay in buffer^*   | –                 |
| D^+(61–85) in H_2O  | +                 |
| D^+(61–84) in HCl    | –                 |

^* Correlates for the entire range tested (Fig. 4).