

Research Article

Proinsulin C-peptide elicits disaggregation of insulin resulting in enhanced physiological insulin effects

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Abstract. Using surface plasmon resonance (SPR) and electrospray mass spectrometry (ESI-MS), proinsulin C-peptide was found to influence insulin-insulin interactions. In SPR with chip-bound insulin, C-peptide mixed with analyte insulin increased the binding, while alone C-peptide did not. A control peptide with the same residues in random sequence had little effect. In ESI-MS, C-peptide lowered the presence of insulin hexamer. The data suggest that C-peptide promotes insulin disaggregation. Insulin/insulin oligomer μM dissociation constants

were determined. Compatible with these findings, type 1 diabetic patients receiving insulin and C-peptide developed 66% more stimulation of glucose metabolism than when given insulin alone. A role of C-peptide in promoting insulin disaggregation may be important physiologically during exocytosis of pancreatic β -cell secretory granules and pharmacologically at insulin injection sites. It is compatible with the normal co-release of C-peptide and insulin and may contribute to the beneficial effect of C-peptide and insulin replacement in type 1 diabetics.

Keywords. Surface plasmon resonance, electrospray ionization mass spectrometry, insulin effect, diabetes type 1, proinsulin C-peptide, insulin disaggregation, insulin hexamer decrease.

C-peptide, the connecting peptide of proinsulin, plays an important role in the biosynthesis of insulin. It serves as a linker between the B and A chains of insulin, facilitating appropriate folding and formation of the insulin SS bridges [1–3]. C-peptide also exerts physiological effects and shows the characteristics of a bioactive peptide. It binds specifically at nanomolar concentrations to cell membranes [4], appears to be associated with G-protein-coupled effects, resulting in activation of Ca^{2+} [5] and MAP-kinase [6–8] -dependent intracellular signalling.

Insulinomimetic effects of C-peptide have also been observed [9]. C-peptide stimulates Na^+, K^+ -ATPase and endothelial nitric oxide synthase activities [10–12]. Glomerular hyperfiltration and albumin excretion in urine of diabetes type 1 patients or in animal models of type 1 diabetes are diminished by C-peptide administration [13, 14]. Likewise, C-peptide therapy results in amelioration or reversal of diabetes-induced functional and structural abnormalities of peripheral nerves [15]. Many molecular interactions and clinical effects of C-peptide have been documented [16, 17]. In spite of all this evidence, an independent action of C-peptide has not been generally accepted. This is explained by several facts, including the

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high species variability of C-peptide in a manner atypical of many hormones [8] and the absence of reports of a receptor for C-peptide.

Recently, we studied molecular interactions between C-peptide and specific proteins using surface plasmon resonance (SPR). The use of SPR in tracing C-peptide binding prompted us to use this technique in studies of C-peptide interactions with insulin. These results, together with mass spectrometric findings and clinical results in patients with type 1 diabetes form the basis of the present report.

Materials and methods

Insulin and C-peptide were covalently linked to Biacore CM5 chips with both N- and C-terminal attachments, following the procedures given by the chip manufacturer. Human insulin (Actrapid, Novo Nordisk, Denmark) was used after desalting on NAP 10 columns. Human C-peptide and scrambled (same composition with different, random sequence) C-peptide were from Sigma Genosys (Cambridge, UK). For the C-terminal attachments, surface thiol coupling up to 5000 response units (RU) was used, and for the N-terminal attachment, amine coupling up to 1000 RU, in both cases with proper deactivations and controls as given by the manufacturer. For comparison, an insulin analogue preparation (Humalog; Eli Lilly, Fegersheim, France) with a residue exchange to give a rapid-acting insulin was used, also after prior desalting on NAP 10 columns.

Interaction analyses were performed with a Biacore 3000 instrument at a flow rate of 20 μ l/min at pH 3–7 (10 mM Na citrate for pH 3, 4 and 5; 10 mM bis-Tris for pH 6; 10 mM Tris/HCl for pH 7), all buffers containing 100 mM NaCl and 0.005% SP20. All Biacore data were analysed with the Bia evaluation software 4.1. Additional models for curve-fit calculations were constructed in IGOR Pro (version 4.01.A, WaveMetrics).

For mass spectrometry, the data were acquired on a Q-TOF Ultima API instrument (Waters, Milford, Mass.) equipped with the standard Z-spray source, operated in the positive-ion mode under the control of MassLynx 4.1 between 700–3500 m/z with a rate of 5 scans/s. Samples were introduced via a nanoflow electrospray interface from metal-coated borosilicate glass capillary needles (Proxeon Biosystems, Odense, Denmark). The source temperature was 80 °C. Conditions were set with a capillary voltage at 1.6 kV, cone and RF Lens 1 energies of 90 and 50 V, respectively, and a source pressure of 6.0×10^{-5} mbar with the use of collision gas. Both insulin and C-peptide solutions were prepared as 600 μ M solutions in H₂O/HCl, pH 5, insulin after desalting as mentioned above, and extensive dialysis against this solution. Different sample concentrations were prepared by dilution of the stock solution in H₂O/HCl, pH 5.

For effects in diabetic patients, nine type 1 diabetes patients (clinical characteristics in Table 1) were studied on 2 separate days with at least 1 week in between, and four patients were studied on a third occasion. Besides insulin they had no medication. All were informed of the purpose, nature and possible risks of the study before giving consent to participate, and the study protocol was approved by an ethics committee. The patients were admitted to the hospital the evening before the study and maintained on intravenous low-dose insulin during the night. The insulin infusion was so adjusted that the blood glucose was in the range of 4–7 mM in the morning. At about 8 a.m., the insulin infusion was stopped and 20 min later a subcutaneous injection of either a mixture of insulin (10 U Humulin; Eli Lilly, Indianapolis, Ind.) and C-peptide (60 nmol, recombinant human C-peptide; Eli Lilly), or insulin (10 U) alone was given in randomized double-blind fashion. On a third occasion, insulin (10 U) and C-peptide (60 nmol) were given simultaneously but in two separate subcutaneous depots, one on each side of the abdomen. The injections were given with an automatic injection device (standardized for speed and

Table 1. Individual clinical data for the patients.

Patient No.	Sex (F, M)	Age (years)	BMI (kg/m ²)	Diabetes duration (years)	Insulin dose (U/kg per 24 hours)	HbA _{1c} ¹ (%)
1	M	46	29.9	31	0.95	5.2
2	M	28	21.9	13	0.78	7.7
3	M	32	24.3	12	0.89	6.8
4	F	29	26.0	15	0.40	9.9
5	F	23	26.7	12	0.76	6.1
6	F	29	20.6	11	0.70	5.8
7	F	35	22.5	22	0.75	5.1
8	F	34	21.2	20	0.80	6.5
9	M	31	26.3	17	0.63	8.3

¹ Reference normal value <4%. All patients were C-peptide negative (<0.20 nmol/l).

depth) 100 mm laterally of the umbilicus into the middle of the subcutaneous tissue layer, the depth of which was determined with ultrasound. Plasma glucose concentrations were measured every 5 min throughout the study. The level was allowed to decrease to 3.5 mM and then maintained in the range 3.5–5.0 mM by variable glucose infusion [18]. Plasma glucose was measured using a glucose oxidase method on a Beckman Glucose Analyzer 2 (Beckman, Fullerton, Calif.). Insulin concentrations were determined using a radioimmunoassay. Standard statistical methods were employed using the Student *t*-test (paired and unpaired) when applicable. Data are presented as average values \pm SE.

Results

Using insulin and C-peptide preparations, we studied the effects of C-peptide on insulin-insulin interactions measured with SPR and electrospray ionization mass spectrometry (ESI-MS). Similarly, with the glucose clamp technique, we evaluated the effects on whole-body glucose utilization by addition of C-peptide to injected insulin in diabetic patients.

SPR analysis of insulin-insulin interactions. Insulin in solution interacts with insulin molecules immobilized on the dextran surface of Biacore CM5 chips. Curves obtained (Fig. 1) are similar, differing only with the attachment loads of the chips used, independent of whether the chip-bound insulin is attached by N- or C-terminal coupling. Insulin-insulin interactions were studied at analyte concentrations of 0.025–10 μ M and were optimal with least background at pH 5. After background subtraction and averaging (over three injections per concentration) of the responses, the sensorgrams fitted best (using least-squares global curve fitting with IGOR Pro; Fig. 1b) to a

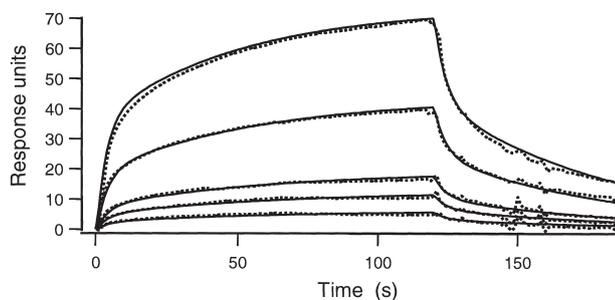


Figure 1. SPR binding curves of insulin with insulin at pH 5 in solution at concentrations of 0.05, 0.1, 0.2, 0.5 and 1 μ M (from bottom to top) over a surface with insulin immobilized by N-terminal (a) and by C-terminal (b) attachment. Each curve is the average of three injections in random order and after blank subtraction (dotted curves). The superimposed curves show the results of global fitting and a perfect match with a model having an analyte with two binding sites and a chip-bound immobilized ligand with one binding site.

model of an analyte with two binding sites competing for one binding site on the ligand. This model applies also for the condition where either analyte or ligand (of one binding site each), due to variations in conformations, give rise to two kinetically distinguishable interaction paths. Accordingly, two apparent equilibrium dissociation constants were obtained, one with a K_D of 2.71 μ M and another with a K_D of 4.65 μ M (with a relatively slow and quick dissociation rate, respectively). Similar attempts at derivation of other binding models (one to one, one to one followed by a conformational change, or a dynamic dimer-binding model) failed to fit the sensorgrams obtained. These results show that the insulin-insulin interactions can be studied by SPR.

The interactions were greatly affected by the pH of the solution and somewhat by the presence of Zn ions (inhibiting at concentrations above 1 \times analyte), or Zn chelators (optimal at EDTA 5 \times analyte concentration, non-saturation at lower excess, and inhibitory at higher excess). Insulin Humalog (Eli Lilly), a human insulin analogue with a structural part exchanged to Lys-Pro (instead of Pro-Lys in insulin) favouring a monomeric nature under physiological conditions [19], was also tested. The analogue showed weak interactions when used as analyte over a surface with the same analogue immobilized. It also gave only little interaction with native insulin, both when the analogue was in the soluble flow-through over human insulin on the chip (N-terminally attached) and when the arrangement was the opposite. This shows that the SPR binding characteristics agree with known properties of human insulin and insulin analogues.

SPR analysis of C-peptide influences on insulin-insulin interactions. Initially, we attempted to find C-peptide-insulin interactions by applying C-peptide or insulin as the surface-bound ligand (attachment in either direction) and the other partner in the analyte position (the flow-through) over a wide concentration range (10 pM to 10 μ M). In no case did we find evidence for binding interactions. These results showed that C-peptide and insulin appear to have no strong binding in the insulin form(s) present on the chip under the SPR conditions. This form(s) is likely to be mainly monomeric for three reasons: first, pretreatments are strongly dissociative; second, ESI-MS (see below) of C-peptide-insulin mixtures show few heterodimers; third, addition of insulin to the analyte in SPR (see below) shows an effect of C-peptide not present without the insulin addition. Hence, C-peptide is concluded to lack a strong binding site for monomeric insulin. This conclusion is compatible with the fact that C-peptide and insulin are joined in proinsulin by covalent bonds rather than binding attachments, and with little ordered structure in the C-peptide part [20, 21]. We also tested binding of C-peptide in solution to chip-bound C-peptide and found no evidence for such bind-

ing either. This appears relevant, since C-peptide is very negative (-5 charges), with high repulsive forces. However, C-peptide, mixed with insulin in solution, influenced the insulin-insulin interactions measured above. A 1- to 5-fold C-peptide excess over insulin analyte increased the observable SPR binding signal (Fig. 2a), and this effect was reproducible over a considerable concentration interval. It was quite specific for C-peptide, not being shown to any appreciable extent by scrambled C-peptide (Fig. 2b). The new curve obtained in the presence of C-peptide did not fit into the standard model fittings (see above). Presumably, therefore, the extra interaction observed in the presence of C-peptide and insulin may involve oligomer states of insulin and binding interactions of C-peptide with such states. Whether the critical C-peptide binding oligomers are insulin hexamers or lower oligomers is difficult to evaluate from just the SPR data. However, the disappearance of insulin hexamers in the presence of C-peptide upon ESI-MS analysis (see below) suggests that C-peptide binding to analyte insulin hexamers in SPR causes their disaggregation and hence an increase in insulin monomers with resulting increased binding to the chip-ligand. An effect via C-peptide binding to insulin dimers appears possible looking just at the

SPR data, but would anyway suggest C-peptide physiological effects via the insulin hexamer since it is a trimer of dimers [22]. In considering insulin-C-peptide binding interactions, segment similarities between C-peptide and parts of insulin [M. Henriksson, J. Johansson, T. Moede, I. Leibiger, J. Shafqat, P. O. Berggren and H. Jörnvall, unpublished data] may be of interest. Time and temperature had some, but limited, effects on the binding curves. Thus, higher temperature ($37\text{ }^{\circ}\text{C}$ instead of $25\text{ }^{\circ}\text{C}$) increased the insulin-insulin binding. C-peptide appeared to slightly reduce the rate of dissociation. In conclusion, C-peptide appears to influence insulin oligomer-forming capacity and to some extent oligomer stability.

We also tested C-peptide analogues. The C-terminal pentapeptide, previously shown to replace C-peptide effects in several assays [4, 5, 7, 8, 10], also stimulated SPR-measurable binding when mixed with insulin in the flow-through solution, while a Glu27Ala C-peptide analogue, inactive in the assays, did not stimulate the SPR signal in the presence of insulin. Two other analogues with Glu to Ala replacements (at positions 3 and 11), previously found to be more active in one assay [8] than the 27 replacement, also gave a greater SPR signal increase with insulin than did the position 27 analogue. In conclusion, much of the C-peptide influence on insulin oligomers seems to be associated with the presence of Glu27, whether in the C-terminal pentapeptide fragment or in the entire C-peptide. Hence, the importance of Glu27 appears to be correlated with the C-peptide effect on the SPR-measurable binding to insulin oligomers.

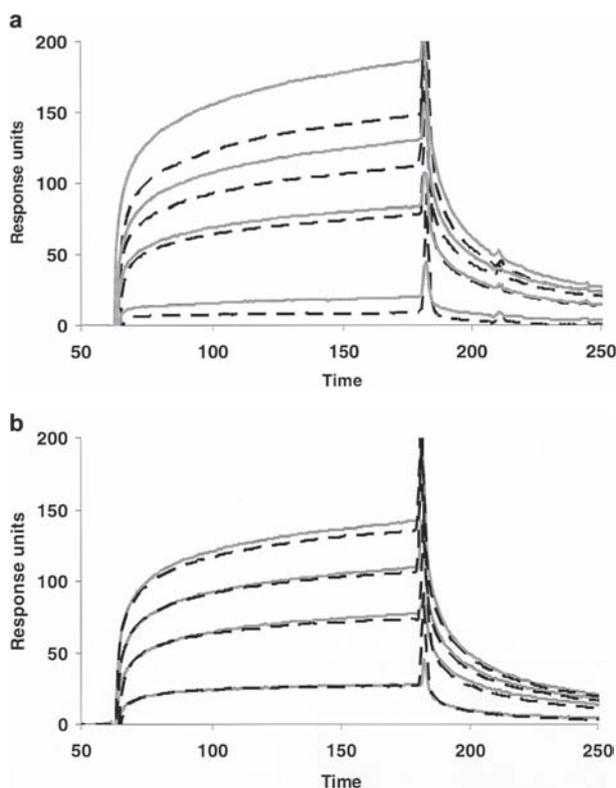


Figure 2. SPR binding curves of insulin at pH 5 alone in solution (dashed lines) and mixed in solution with a 5-fold excess of C-peptide (grey lines) (a) and scrambled C-peptide (b), over a surface with insulin immobilized at the C-terminal. Insulin concentrations used were 1, 5, 10 and $20\text{ }\mu\text{M}$ (from bottom to top), each curve being the average of three runs and after blank subtraction.

C-peptide influences hexamer insulin states observed in ESI-MS analysis. Binding interactions for insulin and other proteins can also be studied by mass spectrometry [23]. We therefore tested the insulin solutions and the insulin/C-peptide mixtures by direct inlet in ESI-MS analysis. The patterns obtained were complex, with a mixture of ion charge states, and in each case with several Na^+ and K^+ adducts, but three observations were clear.

The first was that C-peptide in ESI-MS shows the presence of many ion adducts. This is consistent with its highly negative charge and is also well-known in analyses of other peptides. The adduct formations easily overshadow patterns from the presence of oligomeric forms at low abundance, and the strength of ESI-MS is not to distinguish differential effects between C-peptide and its similarly charged analogues but rather to trace effects on insulin oligomers, especially hexamers. Nevertheless, at low peptide concentrations, C-peptide showed slightly more effects on insulin than scrambled C-peptide. The second observation is that several low-abundance insulin oligomers were observed (dimers, trimers) in addition to the clearer hexamers, and at high C-peptide concentrations, C-peptide dimers and even insulin-C-peptide heterodimers. Third, and most consistent, the presence of hexamer signals at different charge states in the ESI

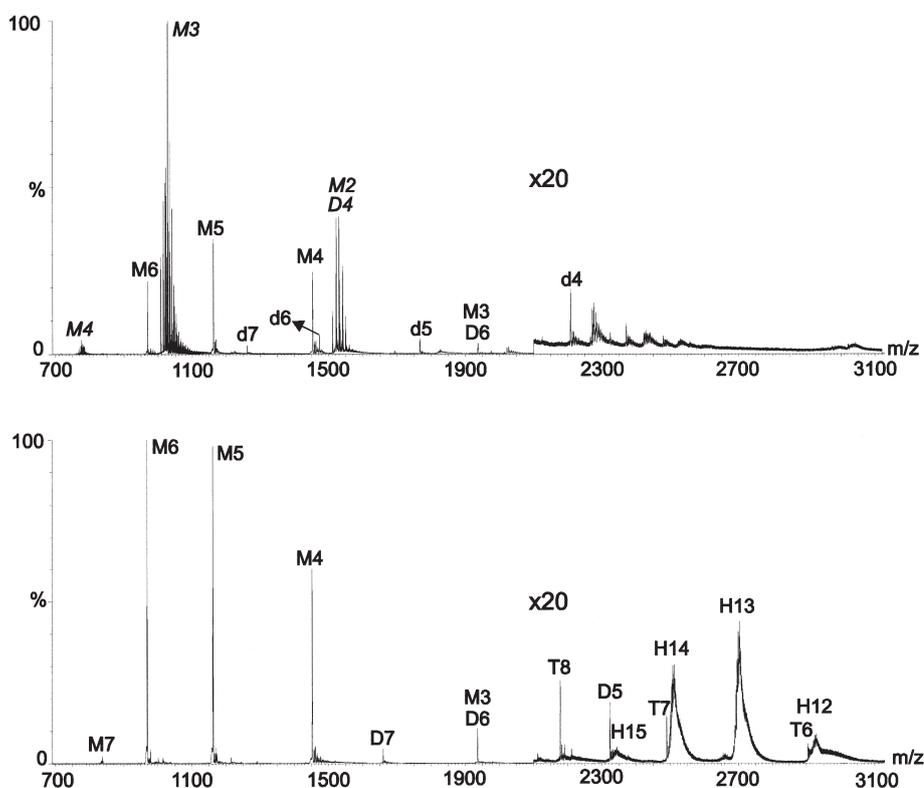


Figure 3. Nano-ES mass spectra of insulin (30 μ M, lower panel) and mixture of insulin and C-peptide at 1:1 ratio (both at 30 μ M, upper panel) at pH 5. The charge states labelled with M, D, T and H in Roman represent identified monomers, dimers, trimers and hexamers, respectively, of insulin, in *italics*, those identified for C-peptide, and in lower case letters, those for a C-peptide-insulin heterodimer. Unlabelled peaks represent variable ion adducts of C-peptide oligomers that were not easily interpreted. The peaks after m/z 2100 are shown at magnification $\times 20$ in both panels. The lower panel shows the presence of insulin hexamers at 12⁺, 13⁺, 14⁺ and 15⁺ charge states, while the upper panel shows their absence at equimolar co-presence of C-peptide.

mass spectra markedly decreased from their level in insulin solutions to an absence in insulin-C-peptide mixtures (Fig. 3). Hence, ESI-MS analysis suggests that C-peptide depolymerizes the hexameric aggregates of insulin.

Effect on glucose utilization of C-peptide additions to insulin injected to type 1 diabetic patients. The insu-

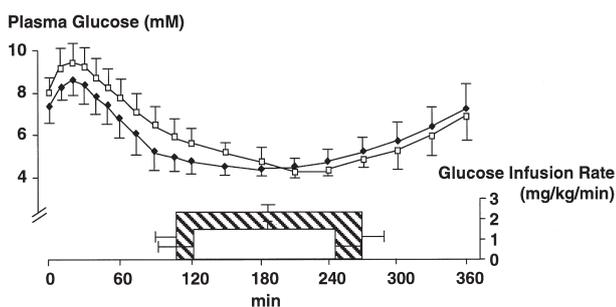


Figure 4. Glucose concentrations (left scale) and glucose infusion rates (right scale) after subcutaneous injection of C-peptide (60 nmol) plus insulin (10 U) (filled diamonds, hatched area) or insulin alone (10 U) (open squares, white area) in nine type 1 diabetes patients. The glucose infusion required to prevent hypoglycaemia was 66% greater ($p < 0.01$) after subcutaneous injection of insulin plus C-peptide compared with insulin alone.

lin-induced increase in whole-body glucose utilization after subcutaneous injection of either insulin plus C-peptide or insulin alone was evaluated using the glucose clamp technique. The combined injection of equimolar amounts of insulin and C-peptide required a glucose infusion that tended to be of longer duration (158 ± 25 vs 123 ± 28 min) and greater magnitude (+66%, $p < 0.01$) than the corresponding values after injection of insulin alone (Fig. 4). In another set of experiments, insulin and C-peptide were injected either in the same or in separate subcutaneous abdominal depots, approximately 20 cm apart. When C-peptide and insulin were administered in the same depot, the reduction in plasma glucose was significantly faster (0–60 min, $p < 0.02$) than when the two were administered in separate depots (Fig. 5a). Insulin appeared more rapidly in the circulation and in larger amounts (AUC 0–360 min, $p < 0.05$) compared with the corresponding data after injection into separate depots (Fig. 5b). The amount of glucose infused to avoid hypoglycaemia had to be increased by 129% ($p < 0.01$) when insulin and C-peptide were administered in the same depot compared with when separate depots were used, and the duration of the infusion was longer (186 ± 24 vs 117 ± 37 min, $p < 0.02$).

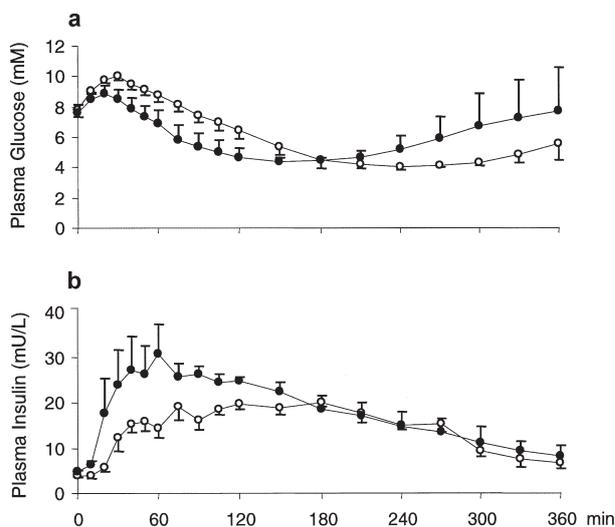


Figure 5. Glucose (a) and insulin (b) concentrations after subcutaneous injection of equimolar amounts of C-peptide (60 nmol) and insulin (10 U) in the same depot (black symbols) or in separate depots (open symbols) in four type 1 diabetes patients.

Discussion

This study measures C-peptide effects on insulin under three highly divergent conditions, in solution versus a solid-phase chip-bound partner, in gas phase, and in patients. Although each set of experiments has restrictions complicating interpretations (different oligomeric states, different phases and in part unknown surface effects in the SPR experiments; unclear significance of gas phase interactions in ESI-MS; non-molecular interaction studies in the patients), all three experimental sets show C-peptide influences on insulin effects. This consistency appears relevant. The combined SPR and ESI-MS data show that C-peptide influences insulin-insulin interactions affecting, in particular, oligomeric states, and in ESI-MS decreasing hexameric signals. As obvious from the presence of several oligomers, there should be many separate interaction constants. Two were estimated by curve fitting to binding models in the SPR experiments, both in the micromolar range. In that range, interactions are not likely to occur in serum or tissues, but are of interest in relation to insulin secretion in the pancreas of healthy individuals and at the injection sites of diabetic subjects. Thus, the possibility should be considered that a physiological effect of C-peptide may be its contribution to the formation of insulin monomers following exocytosis of the secretory granule content of hexameric insulin into the pancreatic extracellular space. Such a C-peptide function is compatible with the physiological co-release of C-peptide and insulin. C-peptide may also affect insulin oligomeric states and disaggregation at subcutaneous sites of insulin injection in diabetic patients. Consequently, we conclude that C-peptide may increase the bioavailability of insulin.

Consistent with this conclusion from the molecular studies, the present patient data show that co-injection of C-peptide and insulin makes insulin appear more rapidly in the circulation and enhances its stimulatory effect on glucose utilization. Combined, all three results are compatible with a previous report concerning insulin and C-peptide co-administration by continuous-rate subcutaneous infusion for 1 month in patients with type 1 diabetes; this resulted in improved glycaemic control and lowered insulin requirements [24]. The possibility may be considered that C-peptide enhances insulin absorption also by stimulation of local nitric oxide release [12], resulting in increased subcutaneous blood flow. A major C-peptide-induced circulatory effect appears less likely, though, since intravenous, as distinct from subcutaneous, co-infusion of insulin and C-peptide in patients with type 1 diabetes results in a more rapid onset and more marked hypoglycaemia than infusion of insulin alone [25]. Thus, the clinical findings appear to provide support for the molecular observations that disaggregation of hexameric insulin is facilitated in the presence of C-peptide, resulting in enhanced insulin action. We conclude from the three sets of studies performed here that C-peptide administered in addition to insulin may be beneficial in the treatment of type 1 diabetes.

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