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Splitting the chains: ultra-basal insulin analog uncovers a redox mechanism of hormone clearance

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Reporting in *Nature Communications*, Kjeldsen and colleagues describe a redox mechanism of insulin clearance based on separation of A- and B chains. Exploiting an ultra-long-acting analog protected from classical clearance pathways, the study highlights principles of protein stability in pharmacology.

The high-resolution crystal structure of insulin[1], a landmark in the history of structural biology, has stimulated continuing efforts to engineer therapeutic analogs with enhanced pharmacologic properties[2]. A major focus has been rational optimization of the pharmacokinetic (PK) properties of insulin analog formulations (whether more rapid-acting or prolonged) to enable safer and more effective management of glycemic control in diabetes mellitus (DM). Understanding structural determinants of insulin stability has been critical to these efforts, as degradation of insulin limits the shelf life of insulin products and makes necessary a complex and costly cold chain of global distribution[3].

A current frontier of insulin pharmacology is defined by the design and clinical application of once-a-week analog formulations[4]. Such "ultra-basal" products, which would match the schedule of once-a-week incretin analogs, could be pertinent to treatment of both Type 1 and Type 2 DM (T1D/T2D)[4]. In a multidisciplinary study Kjeldsen and colleagues have investigated one such analog, designated insulin *icodec*[5]. Its mechanism of protracted action is based on tight binding to serum albumin by a C_{20} acyl adduct[2]. The analog also contains three amino-acid substitutions, two in the B chain introduced to attenuate affinity for the insulin receptor—and hence baseline receptor-mediated clearance[6]—and one in the A chain introduced to augment thermodynamic stability. Because albumin binding delays both receptor-mediated[6,7] and renal clearance[8], the present studies of insulin *icodec* has uncovered a third fundamental mechanism of clearance: thiol-mediated cleavage of insulin's interchain disulfide bridges (cystines B7-A7 and B19-A20 in Fig. 1) on prolonged exposure to redox buffers in plasma, leading to circulating cyclic B chains and isomeric cyclic A chains[5]. The isolated peptides are without hormonal activity. Given the very low concentrations of these peptides in the circulation, the reverse reaction (chain combination) is effectively infeasible. Proteolytic cleavage of the isolated chains in the bloodstream was negligible relative to A-B cleavage.

Why has redox-mediated degradation of insulin in the bloodstream not previously been observed? Kjeldsen and colleagues suggest a straightforward kinetic mechanism: the rapid clearance of native insulin (within 5–10 min[6]) simply does not allow enough time for non-negligible cleavage of disulfide bridges by thiol reagents (such as cysteine and glutathione) in plasma[7]. Rapid receptor-mediated clearance of native insulin notwithstanding, the biological consequences of hormone-receptor engagement last 2–4 h, a reflection of complex post-receptor signaling events[6]. The low intrinsic thermodynamic stability of insulin and its exquisite susceptibility of the A-B monomer to reductive cleavage[3] presumably facilitates physiological intracellular degradation.

The findings of Kjeldsen and colleagues[5] are of both conceptual and translational interest. On the one hand, the uncovered thiol-based degradation mechanism suggests that the circulating insulin monomer is kinetically trapped in its native three-dimensional conformation (Fig. 1). Indeed, the slow timescale of reduction in the bloodstream relative to ordinary mechanisms of clearance enables the native *metastable* disulfide-linked A-B heterodimer (whether injected or secreted by the endocrine pancreas) to engage target cells. The thermodynamic ground state under the redox conditions of plasma comprises the isolated cyclic chains. Thermodynamic stability of the A-B heterodimer is assured within the secretory granules of pancreatic β-cells by zinc-mediated hexamer assembly[1–3]. Such native self-assembly also stabilizes most conventional pharmaceutical formulations[2,3]. Although novel in the context of insulin chemistry, analogous physiological exploitation of kinetically trapped heterodimeric proteins has long been posited in studies of glycoprotein hormones (non-covalent αβ dimers of LH, FSH and βHCG)[9].

On the other hand, observation of reductive cleavage of circulating insulin molecules highlights protein stability as a key design consideration for once-a-week analogs[5]. Kjeldsen and colleagues have thus analyzed potential structural mechanisms of resistance to thiol-mediated cleavage associated with acylation (of Lys^{B29}) and with icodec's three amino-acid substitutions (Tyr^{B16}→His, Phe^{B25}→His and Tyr^{A14}→Glu). Such resistance correlated with structural stability as probed by chemical denaturation assays (ΔG_u or free energies of unfolding). Subtle structural effects were characterized by X-ray crystallography at 2.0-Å resolution. The asymmetric unit contains a novel trimer containing a canonical T_2 dimer (as in the original T_6 [2-Zn] hexamer characterized by Hodgkin and coworkers[1]) and a third distorted T protomer. Although the acyl adduct was not well defined in the electron density, the three substitutions were visualized; each occupies a structural environment similar to the native residue. The investigators ascribe thermodynamic stabilization to two distinct electrostatic features[5]:

(i) Phe^{B25}→His enables the variant side chain to form a hydrogen bond to the side chain of Asn^{A21}, which in turn appears to strengthen a salt bridge between the side chain of Arg^{B22} and the C-terminal carboxylate of the A chain.

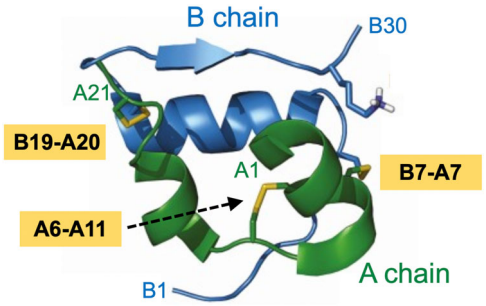

Fig. 1 | Structure of the insulin monomer. Ribbon model of the (crystallographic T state[1]): B chain (30 residues; blue) and A chain (21 residues; green). The native hormone contains two interchain disulfide bridges (cystines B7-A7 and B19-A20; yellow boxes) and one intrachain bridge (cystine A6-A11). Whereas the B7-A7 bridge lies on the surface of the T-state insulin monomer, the other two bridges pack within the hydrophobic core.

(ii) Tyr^{A14} →Glu leads to alignment of Glu^{A14} and Glu^{A17} on one surface of the C-terminal A-chain α-helix, which is proposed to strengthen the native electrostatic interaction between Arg^{B22} and Glu^{A17}.

How Tyr^{B16}→His provides a small additional degree of stabilization was not clear. Of the three substitutions, the stabilizing effect of Glu^{A14} is the most profound.

A limitation of the above structural interpretations is imposed by the acidic conditions of crystallization (pH 4.6) wherein Glu^{A14} and Glu^{A17} are partially protonated. It is therefore not clear how the observed structural relationships may relate to mechanisms of resistance to thiol-mediated cleavage in the bloodstream, i.e., at pH 7.4. Accordingly, it would be of future interest to revisit these inferences through crystallographic or NMR studies at neutral pH. A second limitation pertains to the investigators' emphasis on the nativelike T_2 dimer (irrespective of pH) as a model of the insulin monomer, i.e., the species that is susceptible to thiol attack in the bloodstream. The greater flexibility of the monomer gives rise in molecular dynamics simulation to an ensemble of distorted conformations, including partial folds with enhanced disulfide accessibility[10]. Such conformational excursions (which indeed may have been captured by the crystal structure's third distorted-T protomer) are proposed to accelerate insulin's chemical degradation[3].

The authors note that the stabilizing Tyr^{A14}→Glu substitution has been widely employed in studies of insulin analogs, both basic[11] and translational[2,4]. Its major thermodynamic impact may be unrelated to subtle structural features of the native state. Among structures of native insulin in different crystal forms, Tyr^{A14} projects from the surface of the A chain to occupy a broad range of highly solvent-exposed conformations. It is intriguing to suggest that the Tyr^{A14}→Glu substitution confers a "reverse hydrophobic effect" mediated by relative solvation in the unfolded state[12]. This putative mechanism envisions that Glu^{A14} mitigates a hidden thermodynamic penalty incurred by hyper-exposure of Tyr in the native state relative to the unfolded state.

The marginal stability of insulin's disulfide bridges on prolonged exposure to the redox conditions of plasma, uncovered by Kjeldsen and colleagues in studies of a once-a-week analog[5], comes as a surprise and yet is in accordance with the anomalous instability of native disulfide pairing in insulin-like growth factors (IGFs). Whereas the stability of IGFs is augmented by specific binding proteins, the insulin monomer is protected as a metastable structure within a kinetic trap. Given that physical separation of the oxidized insulin chains in the bloodstream effectively prevents their re-combination, next-generation ultra-stable insulin analogs might exploit a single-chain topology (like IGFs)[11]. Indeed, an SCI moiety is part of an immunoglobulin-related fusion protein in a new once-a-week product (insulin Efsitora alpha; Eli Lilly and Co)[13]; this design may mitigate the risk of redox-related degradation in vivo. Further, given the intrinsic susceptibility of disulfide bonds in insulin (and globular proteins in general) to thiol attack, it is possible that their substitution by diselenide bridges may provide a further route to optimization[14]. Beyond the domain of therapeutic protein engineering, the findings of Kjeldsen and colleagues also raise the question of whether disease-related perturbations in the redox chemistry of blood[15] might be associated with clinical variability in the efficacy of a once-a-week analog. This seminal study thus highlights continuing opportunities for conceptual insight and translational innovation in insulin's second century.

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References

1. Blundell, T. L. et al. Atomic positions in rhombohedral 2-zinc insulin crystals. *Nature* **231**, 506–511 (1971).
2. Jarosinski, M. A. et al. New Horizons: Next-Generation Insulin Analogues: Structural Principles and Clinical Goals. *J. Clin. Endocrinol. Metab.* **107**, 909–928 (2022).
3. Brange, J. & Langkjoer, L. Insulin structure and stability. *Pharm. Biotechnol.* **5**, 315–350 (1993).
4. Rosenstock, J. et al. The Basis for Weekly Insulin Therapy: Evolving Evidence With Insulin Icodec and Insulin Efsitora Alfa. *Endocr. Rev.* **45**, 379–413 (2024).
5. Hubálek, F. et al. Enhanced disulphide bond stability contributes to the once-weekly profile of insulin icodec. *Nat. Commun.* **15**, 6124–6135 (2024).
6. Genuth, S. M. Metabolic clearance of insulin in man. *Diabetes* **21**, 1003–1012 (1972).
7. Flier, J. S. et al. Impaired in vivo insulin clearance in patients with severe target-cell resistance to insulin. *Diabetes* **31**, 132–135 (1982).
8. Chamberlain, M. J. & Stimmler, L. The renal handling of insulin. *J. Clin. Invest.* **46**, 911–919 (1967).
9. Ruddon, R. W. et al. Detection of a glycosylated, incompletely folded form of chorionic gonadotropin beta subunit that is a precursor of hormone assembly in trophoblastic cells. *J. Biol. Chem* **262**, 12533–12540 (1987).
10. Busto-Moner, L., Feng, C. J., Antoszewski, A., Tokmakoff, A. & Dinner, A. R. Structural ensemble of the insulin monomer. *Biochemistry* **60**, 3125–3136 (2021).
11. Glidden, M. D. et al. An ultrastable single-chain insulin analog resists thermal inactivation and exhibits biological signaling duration equivalent to the native protein. *J. Biol. Chem.* **293**, 47–68 (2018).
12. Pakula, A. A. & Sauer, R. T. Reverse hydrophobic effects relieved by amino-acid substitutions at a protein surface. *Nature* **344**, 363–364 (1990).
13. Wysham, C. et al.; for the QWINT-2 Investigators. Insulin Efsitora versus Degludec in Type 2 Diabetes without Previous Insulin Treatment. *N. Engl. J. Med.* https://doi.org/10.1056/NEJMoa2403953 (2024). online ahead of print.
14. Weil-Ktorza, O., Dhayalan, B., Chen, Y. S., Weiss, M. A. & Metanis, N. Se-Glargine: Chemical Synthesis of a Basal Insulin Analogue Stabilized by an Internal Diselenide Bridge. *Chembiochem.* **25**, e202300818 (2024).
15. Oliveira, P. V. S. & Laurindo, F. R. M. Implications of plasma thiol redox in disease. *Clin. Sci. (Lond)* **132**, 1257–1280 (2018).

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