HHS Public Access

Author manuscript

Chemistry. Author manuscript; available in PMC 2021 May 28.

Published in final edited form as:

Chemistry. 2020 April 09; 26(21): 4695–4700. doi:10.1002/chem.202000309.

Reassessment of an Innovative Insulin Analogue Excludes Protracted Action yet Highlights Distinction between External and Internal Diselenide Bridges

Balamurugan Dhayalan[a], **Yen-Shan Chen**[a], **Nelson B. Phillips**[b], **Mamuni Swain**[b], **Nischay Rege**[b], **Ali Mirsalehi**[b], **Mark Jarosinski**[a], **Faramarz Ismail-Beigi**[b], **Norman Metanis**[c], **Michael A. Weiss**[a],[d]

[a]Department of Biochemistry & Molecular Biology, Indiana University School of Medicine, Indianapolis, IN 46202, USA

[b]Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106, USA.

[c]The Institute of Chemistry, The Hebrew University of Jerusalem, Edmond J. Safra, Givat Ram, Jerusalem 91904, Israel

[d]Department of Chemistry, Indiana University, Bloomington, IN 47405, USA

Abstract

Long-acting insulin analogues represent the most prescribed class of therapeutic proteins. An innovative design strategy was recently proposed: diselenide substitution of an external disulfide bridge. This approach exploited the distinctive physicochemical properties of selenocysteine (U). Relative to wild type (WT), Se-insulin[C7U^{A}, C7U^{B}] was reported to be protected from proteolysis by insulin-degrading enzyme (IDE), predicting prolonged activity. Because of this strategy's novelty and potential clinical importance, we sought to validate these findings and test their therapeutic utility in an animal model of diabetes mellitus. Surprisingly, the analogue did not exhibit enhanced stability, and its susceptibility to cleavage by either IDE or a canonical serine protease (glutamyl endopeptidase Glu-C) was similar to WT. Moreover, the analogue's pharmacodynamic profile in rats was not prolonged relative to a rapid-acting clinical analogue (insulin lispro). Although [C7U^{A}, C7U^{B}] does not confer protracted action, nonetheless its comparison to internal diselenide bridges promises to provide broad biophysical insight.

Keywords

Oxidative protein folding; insulin; selenocysteine; selenoprotein; chemical protein synthesis

weissma@iu.edu.

The senior author dedicates this article to the memory of the late Prof. C.M. Dobson (Cambridge University).

Conflict of interest

M.A.W. has equity in Thermalin, Inc. (Cleveland, OH) where he serves as Chief Innovation Officer; he has also been consultant to Merck Research Laboratories and DEKA Research & Development Corp. N.B.P. and F.I.-B. are consultants to Thermalin, Inc. and also have options, warrants or equity. F.I.-B. is a consultant to Sanofi and has received grants from Novo-Nordisk. M.A.W. and N.M. have submitted a provisional patent application to the United States Patent Office regarding internal diselenide bridges in insulin analogues.

Selenocysteine (Sec, U), a near isostere of Cys and the 21st-proteinogenic amino acid,[1] provides an elegant tool in peptide chemistry.[2] Conserved within the active sites of key redox enzymes,[3] the selenol group of Sec has a lower pK_a (near 5.2)[4] and lower reduction potential (E_0= −388 mV) than does the thiol group of Cys. These properties have also been exploited in protein engineering as pairwise Cys-to-Sec substitutions can enhance the rate and efficiency of oxidative folding.[5] Indeed, because diselenide bonds are more stable than disulfides (relative to their respective reduced forms), strategic Sec substitutions can improve protein folding efficiency even *via* non-native pairing when followed by structure-driven rearrangement.[5c, 6]

In this *Communication* we reassess an intriguing proposal by Iwaoka and colleagues that pairwise substitution of an external disulfide bridge in insulin (Cys^{A7} and Cys^{B7}) by Sec provides a novel mechanism of stabilization and protracted action.[7] Long-acting insulin analogues are a mainstay in the treatment of diabetes mellitus (DM), whose global prevalence is approaching pandemic status.[8] The limited shelf life of available insulins above room temperature necessitates a complex and costly cold chain of distribution and storage,[9] a restriction that poses a major challenge in global health.[10]

Two principles underlie the protracted action of current basal insulin products: (i) stabilization of a subcutaneous (SQ) hormone depot and (ii) albumin binding in the bloodstream. The first mechanism is exemplified by insulin *glargine*, the active ingredient of Lantus® (Sanofi): on its SQ injection in a soluble acidic formulation, the protein precipitates at the neutral pH of interstitial fluid. Isoelectric precipitation creates a long-lived depot with slow release of the analogues.[11] The second mechanism is exemplified by insulin *detemir*, the active ingredient of Levemir® (Novo-Nordisk): acylation of a unique lysine at the periphery of the receptor-binding surface (Lys^{B29}) confers albumin binding and hence delays clearance of a circulating depot.[12] A second-generation basal insulin analogue formulation (insulin *degludec*, the active ingredient of Tresiba®; Novo Nordisk) combines these mechanisms as its novel Lys^{B29} adduct (hexadecanedioic acid via a γ-L-glutamyl spacer) was fortuitously found to mediate both SQ polymerization and albumin binding in the bloodstream.[13]

Insulin contains three disulfide bridges, two between chains (A7–B7 and A20–B19), and one within the A chain (A6–A11) (Fig. 1a). Bovine Se-insulin, in which Cys^{A7} and Cys^{B7} were substituted with Sec, was observed to form an external diselenide bridge as a near-isosteric replacement of the canonical A7–B7 disulfide bridge,[7] a conserved feature of vertebrate insulins[14] (as well as insulin-like growth factors [IGFs][15] and relaxins[16]). Arai et al. (2017) demonstrated that this diselenide bridge preserved biological activity in a cellular assay but seemed to protect the analogue from in vitro degradation by insulin-degrading enzyme (IDE), a large metalloprotease with internal substrate-binding chambers (Fig. 1b, c) .[17] Indeed, IDE-catalyzed degradation of bovine Se-insulin[$C7U^A$, $C7U^B$] was reported to be eightfold slower than that of unmodified bovine insulin. Such protection was ascribed to the intrinsic stability of the diselenide bond and possible adjustments in local protein conformation due to the larger size of the selenium atom relative to sulfur. This innovative approach thus sought to exploit fundamental aspects of chalcogen chemistry[3] in an effort to enhance key pharmacologic and biophysical properties of a therapeutic protein.[7] In

addition to their structural interest, the studies of Iwaoka and colleagues appeared to provide an orthogonal and general third principle of protracted action–combinable with known approaches to optimize “third-generation” insulin analogues.[7] These studies employed bovine insulin as a synthetic model.

Because of the prior study’s potential translational impact, we prepared bovine Se-insulin[C7U^{A}, C7U^{B}] and native bovine insulin (using a different synthetic strategy, see scheme 1) to enable their comparative study in vitro and in vivo. In general accordance with the findings of Arai et al. (2017),[7] biological activity was maintained in cell culture. In accordance with the native-like crystal structure of bovine Se-insulin[C7U^{A}, C7U^{B}] reported in their study,[7] including at its receptor-binding surface, the affinity of the analogue for the lectin-purified insulin receptor was similar to that of bovine insulin. Surprisingly, however, chemical denaturation studies indicated that the thermodynamic stabilities of the two proteins were essentially identical. Moreover, only marginal differences were observed in their relative rates of proteolytic digestion by insulin-degrading enzyme (human or rat IDE) and by glutamyl endopeptidase Glu-C.[18] Further, we found that the two proteins exhibited indistinguishable pharmacodynamic (PD) properties on intravenous (IV) or subcutaneous (SQ) bolus injection in a rat model of DM. The present reassessment therefore suggests that substitution of a disulfide bridge on the surface of insulin by a diselenide bridge preserves–but does not enhance–the structure, function, and pharmacologic properties of the hormone.

Insulin contains two chains: an A chain (21 residues) and B chain (30 residues) (Fig. 1a).[19] Se-insulin[C7U^{A}, C7U^{B}] was prepared by Arai et al. through chain combination.[7] The standard protocol was modified to employ 2-aminoethyl methanethiosulfonate (AEMTS) to capture free thiols. Although in our hands bovine A-chain[C7U^{A}] could be made without use of this reagent, synthesis of B-chain[C7U^{B}] exhibited lower yields due to poor solubility. To circumvent this issue, our chemical syntheses exploited a single-chain precursor (“DesDi”) with enhanced oxidative folding properties as described by DiMarchi and colleagues.[20] This 49-residue template (lacking residues B29-B30 and containing a peptide linkage between Lys^{B28} and Gly^{A1}) was modified to incorporate bovine residues Ala^{A8} and Val^{A10} (*versus* human residues Thr^{A8} and Ile^{A10}). Digestion of the respective precursors with trypsin provided corresponding fragments *des*-octapeptide[B23-B30]-bovine insulin (DOI) and *des*-octapeptide[B23-B30]-bovine Se-insulin[C7U^{A}, C7U^{B}] (Se-DOI). The mature products, bovine insulin and Se-insulin[C7U^{A}, C7U^{B}], were obtained by trypsin-mediated semi-synthesis[21] using octapeptide GFFYTPK**A** in which the Lys (underlined) was protected by a Boc group (side chain *t*-Bu protecting groups on Tyr and Thr were also left as such for convenience) and the C-terminal Ala provides bovine residue Ala^{B30} (*versus* Thr^{B30} in human insulin). This strategy is outlined in scheme 1 and documented by HPLC chromatograms (SI Fig. S1 and S2). Although final yields (relative to the crude single-chain precursor) were lower than provided by insulin chain combination (SI Table S1), this protocol can in principle expedite preparation of several analogues from the same Se-DOI batch via semi-syntheses with different B23-B30 octapeptides (see shaded box in SI Scheme S1).[22]

The conformation of bovine Se-insulin[C7U^{A}, C7U^{B}] is similar to that of the parent protein as assessed by far-ultraviolet circular dichroism (Fig. 2a). Deconvolution of respective

spectra yielded estimated α-helix contents of 46.5% (parent) and 40.9% (Se-analogue). Thermodynamic stabilities were probed by chemical denaturation as monitored by ellipticity at helix-sensitive wavelength 222 nm.[22] The corresponding profiles were essentially identical (Fig. 2b). Application of a two-state thermodynamic model[23] implied indistinguishable estimates of free energies of unfolding ($\Delta\Delta G_u$; Table 1).

Stabilities were further interrogated by monitoring respective rates of cleavage of bovine insulin or its Se-insulin[C7U^A, C7U^B] analogue using human IDE (Fig. 3a; corresponding to the studies of Arai et al.)[7] and rat IDE (Fig. 3b; to correlate with our rat studies; below). Equal concentrations of protein were treated separately at 30 °C in Tris-HCl buffer pH 8.0.[7] The protocol was essentially as described in the original study[7] (SI Methods), except that higher protein concentrations were used to enhance HPLC sensitivity (20 μM *versus* 2 μM). With either human or rat IDE, cleavage of the modified proteins was only 25–30% slower than WT (SI Fig. S3). These observations differ from the eightfold (800%) effect reported by Arai et al.[7] but are in accordance with the crystal structure of IDE[24] and its mode of substrate binding[25]: molecular modelling suggests that a diselenide bridge could readily be accommodated within the catalytic chambers (Fig. 1c).[26] We sought to explore whether a simple error in pH might have accounted for the eightfold effect reported by Arai et al.[7] Control studies in which the pH was mis-adjusted from pH 8.0 to pH 6.0 only resulted in a twofold retardation of IDE cleavage, not the eightfold effect reported (SI Fig. S4). It is possible that unknown differences in salts or temperature could together have given rise to a spurious eightfold effect.

Respective rates of cleavage were also evaluated by endopeptidase Glu-C (V8 protease), a canonical serine protease.[22b] Equal concentrations of bovine Se-insulin[C7U^A, C7U^B] and unmodified bovine insulin were treated separately with V8 protease at 37 °C in an ammonium buffer (SI Fig. S5).[27] At successive time points, small aliquots were placed on ice and quenched with 0.1% TFA in 6 M GuHCl, and the digestion process analyzed by HPLC. As expected, four fragments were obtained in each case as characterized by ESI-MS (SI Fig. S6 and S7).[28] Rates of cleavage were similar in the modified and unmodified proteins in accordance with their similar ΔG_u values. This finding suggests that the external diselenide bridge does not constrain segmental conformational fluctuations in bovine insulin —near or far from the sites of A7–B7 modification—that might enhance exposure of the scissile peptide bonds (at Glu^{A4}, Glu^{A17}, Glu^{B13} and Glu^{B21}) to the endopeptidase.

The affinity of bovine Se-insulin[C7U^A, C7U^B] for the lectin-purified human insulin receptor (isoform B as immobilized in a competitive-displacement plate assay[22b]) was similar to that of bovine insulin (Fig. 4). Any difference between respective dissociation constants (130(±20) and 110(±10) pM) was within the error of measurement. Likewise indistinguishable were respective human cell-signaling assays of protein phosphorylation and gene regulation (SI Fig. S8 and S9) at 10 nM hormone concentration. These findings corroborate the cellular studies of Arai et al. obtained at a hormone concentration of 1 μM.[7]

Finally, the biological activity and duration of action of the insulin analogues were evaluated in male Lewis rats rendered diabetic by pancreatic β-cell toxin streptozotocin.[29] The mean animal mass was *ca.* 300 grams; glycemia was elevated to a mean concentration *ca.* 22.2

mM (400 mg/dl). Studies were conducted following subcutaneous (SQ) injection (Fig. 5a,c) and intravenous (IV) bolus injection (Fig. 5b,d). By either route the potency of bovine Se-insulin[$C7U^A$, $C7U^B$] was similar to that of the unmodified hormone. Its duration of action was not extended; indeed, a trend was observed toward foreshortened action, most clearly seen in the normalized intravenous time course of glycemic recovery (Fig. 5d; p-value <0.2). Analyses of these pharmacodynamic profiles are given in the SI Methods and Fig. S10–S12.

Concluding Remarks

The present study provides a reassessment of the proposal by Arai et al. that an external diselenide bridge in insulin provides a novel route to long-acting insulin analogues.[7] Containing substitutions [$C7U^A$, $C7U^B$], the insulin analogue was reported to exhibit marked resistance to degradation by IDE in vitro (relative to bovine insulin). Our results provide evidence that this is not the case and further that insulin action is not prolonged in an animal model of diabetes mellitus. The biophysical, biochemical and biological properties of bovine Se-insulin[$C7U^A$, $C7U^B$] are similar to those of bovine insulin. Such similarity is in accordance with the native-like crystal structure also reported by Arai et al.[7]

It is of interest to compare and contrast the effects of the present *external* diselenide bridge in insulin with those of an *internal* diselenide bridge.[22b] As recently described by Weil-Ktorza et al., pairwise substitution of Cys^{A6} and Cys^{A11} by Sec gives rise to an insulin analogue with enhanced thermodynamic stability and relative resistance to cleavage by endoproteinase V8.[22b] Why would modification of an internal bridge stabilize the protein, but not the corresponding modification of an external bridge? Whereas Sec is often discussed in reference to its altered redox properties (relative to Cys) and whereas the diselenide bridge is discussed in relation to strength of the Se-Se bond (relative to S-S), we envision that the critical factor operative here is the efficiency of core packing. A diselenide bridge is 10–15% larger in length and volume than is a disulfide bridge. Because the native A6–A11 bridge fortuitously resides in a portion of the insulin molecule notable for small gaps between side chains, we propose that the modified bridge enhances local packing, possibly optimizing chalcogen bonds[30] and mitigating the thermodynamic costs of micro-cavities[31] in the native core.[32] The empirical free-energy scale of cavity penalties with the cores of globular proteins[32] implicitly includes effects of entropy-enthalpy compensation (EEC); the latter may in part mitigate stabilization via enhanced van der Waal interactions and chalcogen bonds (favourable terms in $\Delta\Delta H$) due to damping of neighbouring conformational fluctuations (unfavourable terms in $\Delta\Delta S$).

With an external bridge, the structure of the hydrophobic core would not directly pertain. In particular, because the stronger Se-Se bond would be present in both the folded and unfolded states, its intrinsic stability (relative to S-S) would make no *net* contribution to the change in free energy on unfolding (ΔG_u). It would be of future interest to investigate whether (and how) an external diselenide bridge might alter the dynamics of a protein. Physical situations may exist in which nonlocal dynamic effects of a “stiffer” bridge give rise to complex changes in thermodynamic driving forces, including changes in solvation entropies and enthalpies. The resulting EEC may give rise to net stabilization or destabilization.

Returning to insulin pharmacology, we note in passing that the aforementioned A6-A11 diselenide bridge in human insulin[22b] delays IDE digestion to an extent similar to Se-insulin[C7U^{A}, C7U^{B}] (SI Fig. S13). In neither case was prolonged in vivo activity observed (SI Fig. S14). Despite such lack of relevance to the translational goal of protracted action, it is possible that the augmented thermodynamic stability of Se-insulin[C6U^{A}, C11U^{A}] may enhance the shelf life of pharmaceutical formulations.

In summary, the present reassessment suggests that the goal of designing a novel insulin analogue *that is selectively resistant to IDE degradatio*n remains as a compelling challenge in chemical biology. Although whether IDE is indeed a valid target for diabetes-specific drug design is uncertain,[33] such an analogue could provide an elegant reagent with which to test the physiologic hypothesis that IDE regulates the duration of insulin action—a key issue at the frontier of molecular endocrinology. The structural complexity of IDE's internal catalytic chambers[26] highlights the subtlety of this challenge in protein design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank members of our respective laboratories for discussion; Dr. Y. Yang for preliminary NMR studies; Dr. J. Whittaker and L. Whittaker for advice regarding IR-binding assays; O. Weil-Ktorza for advice regarding protease assays and communication of results prior to publication, and Mads Krogsgaard Thomsen (Novo Nordisk) for the gift of radio-labeled insulin. N.M. acknowledges the financial support of Israel Science Foundation (783/18) and ICRF Acceleration Grant; M.A.W., N.B.P., and F.I.-B. acknowledges the support of the U.S. National Institutes of Health (NIH R01 DK040949). N.R. is a Predoctoral Fellow of the Medical Scientist Training Program at CWRU (NIH 5T32GM007250-38 and NIH Fellowship 1F30DK112644).

References

[1]. Stadtman TC, Ann. Rev. Biochem 1996, 65, 83–100. [PubMed: 8811175]

[2]. Dawson PE, Israel J Chem. 2011, 51, 862–867.

[3]. Metanis N, Hilvert D, Curr. Opin. Chem. Biol 2014, 22, 27–34. [PubMed: 25261915]

[4]. Huber RE, Criddle RS, Arch. Biochem. Biophy 1967, 122, 164–173.

[5]. a)Armishaw CJ, Daly NL, Nevin ST, Adams DJ, Craik DJ, Alewood PF, J. Biol. Chem 2006, 281, 14136–14143; [PubMed: 16500898] b)Gowd KH, Yarotskyy V, Elmslie KS, Skalicky JJ, Olivera BM, Bulaj G, Biochemistry 2010, 49, 2741–2752; [PubMed: 20175537] c)Metanis N, Hilvert D, Angew. Chem. Int. Ed 2012, 51, 5585–5588;d)Dery L, Reddy PS, Dery S, Mousa R, Ktorza O, Talhami A, Metanis N, Chem. Sci 2017, 8, 1922–1926. [PubMed: 28451306]

[6]. Pegoraro S, Fiori S, Cramer J, Rudolph-Bohner S, Moroder L, Protein Sci. 1999, 8, 1605–1613. [PubMed: 10452604]

[7]. Arai K, Takei T, Okumura M, Watanabe S, Amagai Y, Asahina Y, Moroder L, Hojo H, Inaba K, Iwaoka M, Angew. Chem. Int. Ed 2017, 56, 5522–5526.

[8]. van Dieren S, Beulens JW, van der Schouw YT, Grobbee DE, Neal B, Eur. J. Cardiovasc. Prev. Rehabil 2010, 17 Suppl 1, S3–8. [PubMed: 20489418]

[9]. Guthridge SL, Miller NC, Aust. N. Z. J. Public Health 1996, 20, 657–660. [PubMed: 9117976]

[10]. Weiss MA, J. Health Spec 2013, 1, 59–70.

[11]. Campbell RK, White JR, Levien T, Baker D, Clin. Ther 2001, 23, 1938–1957. [PubMed: 11813930]

[12]. Goldman-Levine JD, Lee KW, Ann. Pharmacother 2005, 39, 502–507. [PubMed: 15657117]

[13]. Kalra S, Baruah MP, Niazi AK, Recent Pat. Endocr. Metab. Immun. Drug Discov 2012, 6, 18–23.

[14]. a)Baker EN, Blundell TL, Cutfield JF, Cutfield SM, Dodson EJ, Dodson GG, Hodgkin DM, Hubbard RE, Isaacs NW, Reynolds CD, Philos. Trans. R. Soc. Lond. B Biol. Sci 1988, 319, 369–456; [PubMed: 2905485] b)Conlon JM, Amer. Zool 2000, 40, 200–212.

[15]. a)Nagamatsu S, Chan SJ, Falkmer S, Steiner DF, J. Biol. Chem 1991, 266, 2397–2402; [PubMed: 1989990] b)Upton Z, Francis GL, Chan SJ, Steiner DF, Wallace JC, Ballard FJ, Gen. Comp. Endocrin 1997, 105, 79–90.

[16]. Wilkinson TN, Speed TP, Tregear GW, Bathgate RA, BMC Evol. Biol 2005, 5, 14. [PubMed: 15707501]

[17]. a)Hersh L, Cell. Mol. Life Sci 2006, 63, 2432–2434; [PubMed: 16952049] b)Grune T, Cell Cycle 2015, 14, 2553–2553. [PubMed: 26066350]

[18]. Demidyuk IV, Chukhontseva KN, Kostrov SV, Acta Nat. 2017, 9, 17–33.

[19]. Ryle AP, Sanger F, Smith LF, Kitai R, Biochem. J 1955, 60, 541–556. [PubMed: 13249947]

[20]. Zaykov AN, Mayer JP, Gelfanov VM, Dimarchi RD, ACS Chem. Biol 2014, 9, 683–691. [PubMed: 24328449]

[21]. Inouye K, Watanabe K, Morihara K, Tochino Y, Kanaya T, Emura J, Sakakibara S, J. Am. Chem. Soc 1979, 101, 751–752.

[22]. a)Pandyarajan V, Phillips NB, Cox GP, Yang Y, Whittaker J, Ismail-Beigi F, Weiss MA, J. Biol. Chem 2014, 289, 23367–23381; [PubMed: 24993826] b)Weil-Ktorza O, Rege N, Lansky S, Shalev DE, Shoham G, Weiss MA, Metanis N, Chem. Eur. J 2019, 25, 8513–8521. [PubMed: 31012517]

[23]. Sosnick TR, Fang X, Shelton VM, Methods Enzymol. 2000, 317, 393–409. [PubMed: 10829292]

[24]. Shen Y, Joachimiak A, Rosner MR, Tang WJ, Nature 2006, 443, 870–874. [PubMed: 17051221]

[25]. Manolopoulou M, Guo Q, Malito E, Schilling AB, Tang WJ, J. Biol. Chem 2009, 284, 14177–14188. [PubMed: 19321446]

[26]. Zhang Z, Liang WG, Bailey LJ, Tan YZ, Wei H, Wang A, Farcasanu M, Woods VA, McCord LA, Lee D, Shang W, Deprez-Poulain R, Deprez B, Liu DR, Koide A, Koide S, Kossiakoff AA, Li S, Carragher B, Potter CS, Tang WJ, Elife 2018, 7, e33572. [PubMed: 29596046]

[27]. Vestling MM, J. Chem. Ed 1991, 68, 958–960.

[28]. Thalluri K, Kou B, Gelfanov V, Mayer JP, Liu F, DiMarchi RD, Org. Lett 2017, 19, 706–709. [PubMed: 28124920]

[29]. Yang Y, Petkova A, Huang K, Xu B, Hua QX, Ye IJ, Chu YC, Hu SQ, Phillips NB, Whittaker J, Ismail-Beigi F, Mackin RB, Katsoyannis PG, Tycko R, Weiss MA, J. Biol. Chem 2010, 285, 10806–10821. [PubMed: 20106984]

[30]. a)Bortoli M, Ahmad SM, Hamlin TA, Bickelhaupt FM, Orian L, Phys Chem Chem Phys 2018, 20, 27592–27599; [PubMed: 30371693] b)Scilabra P, Terraneo G, Resnati G, Acc. Chem. Res 2019, 52, 1313–1324. [PubMed: 31082186]

[31]. Tilton RF, Kuntz ID, Petsko GA, Biochemistry 1984, 23, 2849–2857. [PubMed: 6466620]

[32]. a)Eriksson AE, Baase WA, Zhang XJ, Heinz DW, Blaber M, Baldwin EP, Matthews BW, Science 1992, 255, 178–183; [PubMed: 1553543] b)Bueno M, Cremades N, Neira JL, Sancho J, J. Mol. Biol 2006, 358, 701–712. [PubMed: 16563433]

[33]. a)Deprez-Poulain R, Hennuyer N, Bosc D, Liang WG, Enee E, Marechal X, Charton J, Totobenazara J, Berte G, Jahklal J, Verdelet T, Dumont J, Dassonneville S, Woitrain E, Gauriot M, Paquet C, Duplan I, Hermant P, Cantrelle FX, Sevin E, Culot M, Landry V, Herledan A, Piveteau C, Lippens G, Leroux F, Tang WJ, van Endert P, Staels B, Deprez B, Nat. Commun 2015, 6, 8250; [PubMed: 26394692] b)Pivovarova O, Hohn A, Grune T, Pfeiffer AF, Rudovich N, Ann. Med 2016, 48, 614–624; [PubMed: 27320287] c)Li H, Wu J, Zhu L, Sha L, Yang S, Wei J, Ji L, Tang X, Mao K, Cao L, Wei N, Xie W, Yang Z, Biosci. Rep 2018, 38.

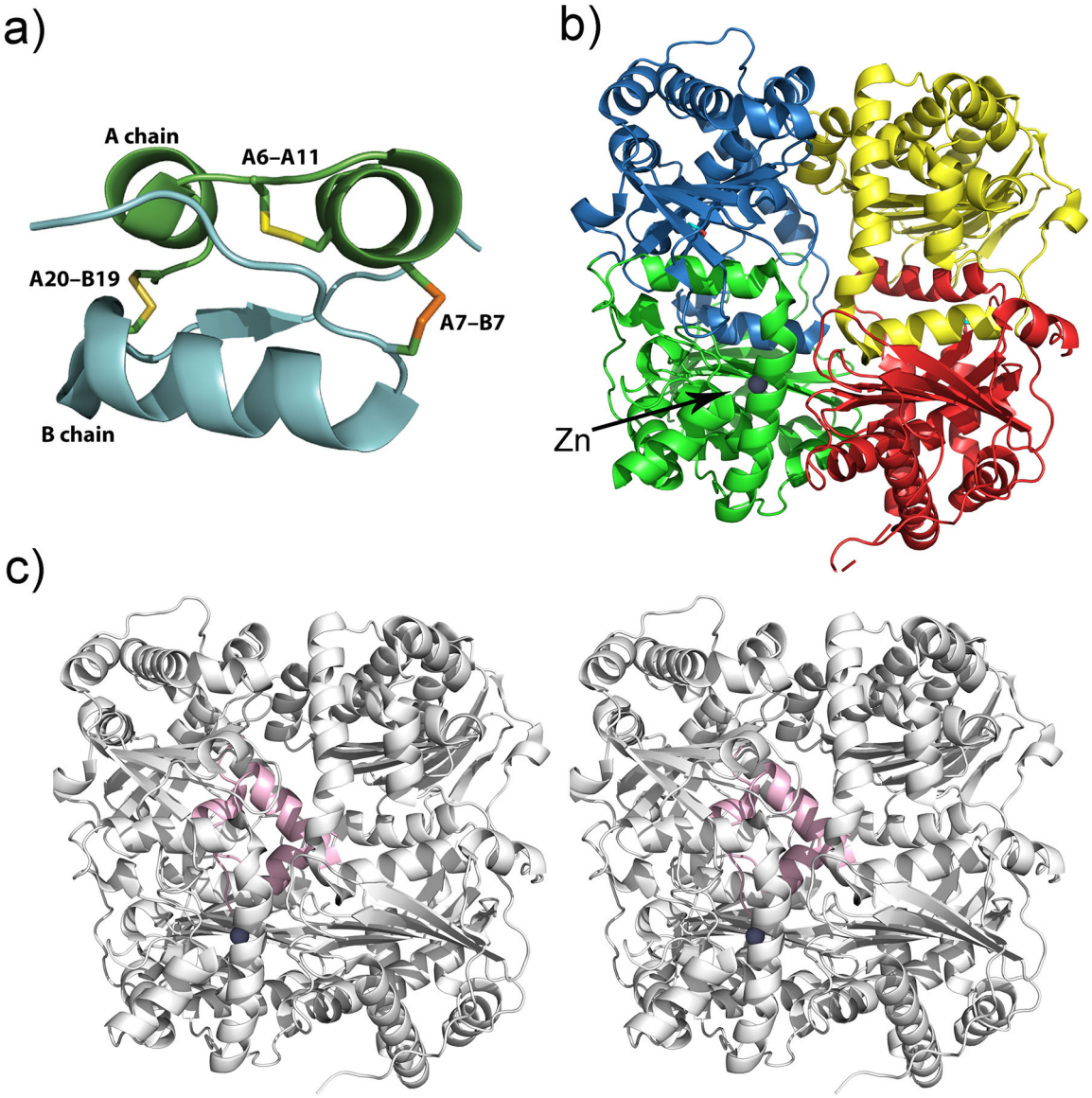


Figure 1.

Structural features of insulin and IDE. **a**. 3-D structure of insulin contains α-helical subdomain stabilized by three disulfide bonds: A6–A11, A7–B7 and A20–B19 as indicated as sticks (PDB: 3w7y). **b**. Ribbon diagram of substrate free-IDE (PDB: 2jg4). **c**. Stereo view of IDE-insulin complex (PDB: 2wby). IDE-bound insulin molecule shown in pink.

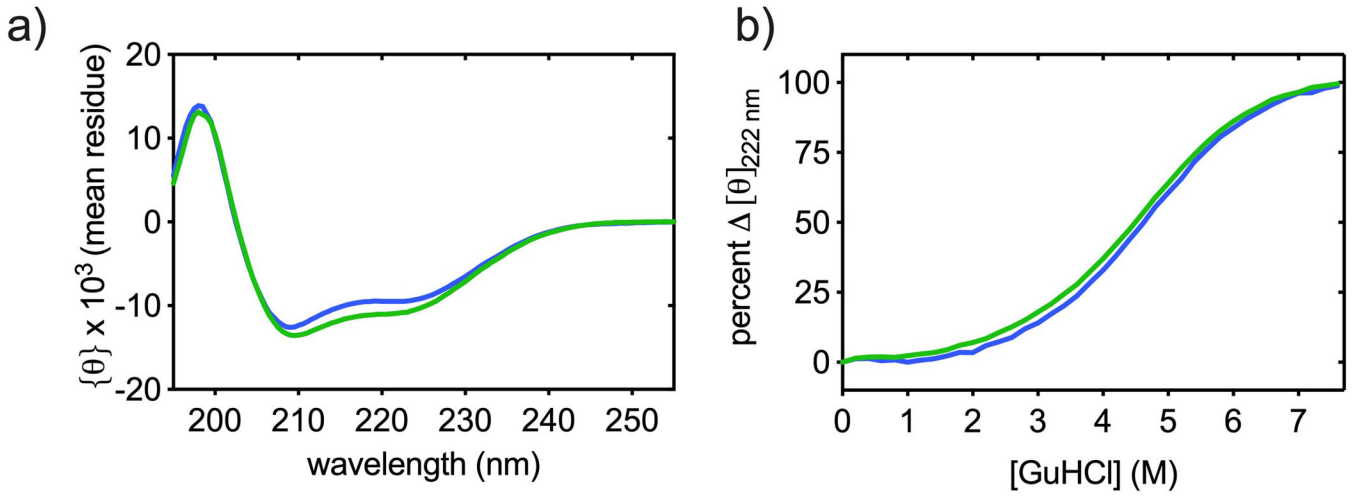


Figure 2.

Structural characterization and thermodynamic stability of insulin analogues. **a**. CD spectra of Se-bovine insulin[C7U^{A}, C7U^{B}] (Se-Ins, *blue*) and bovine insulin (*green*). **b**. Guanidine denaturation assays of insulin analogues monitored by ellipticity at 222 nm; color code as in panel a. The resulting stability values are given in Table 1.

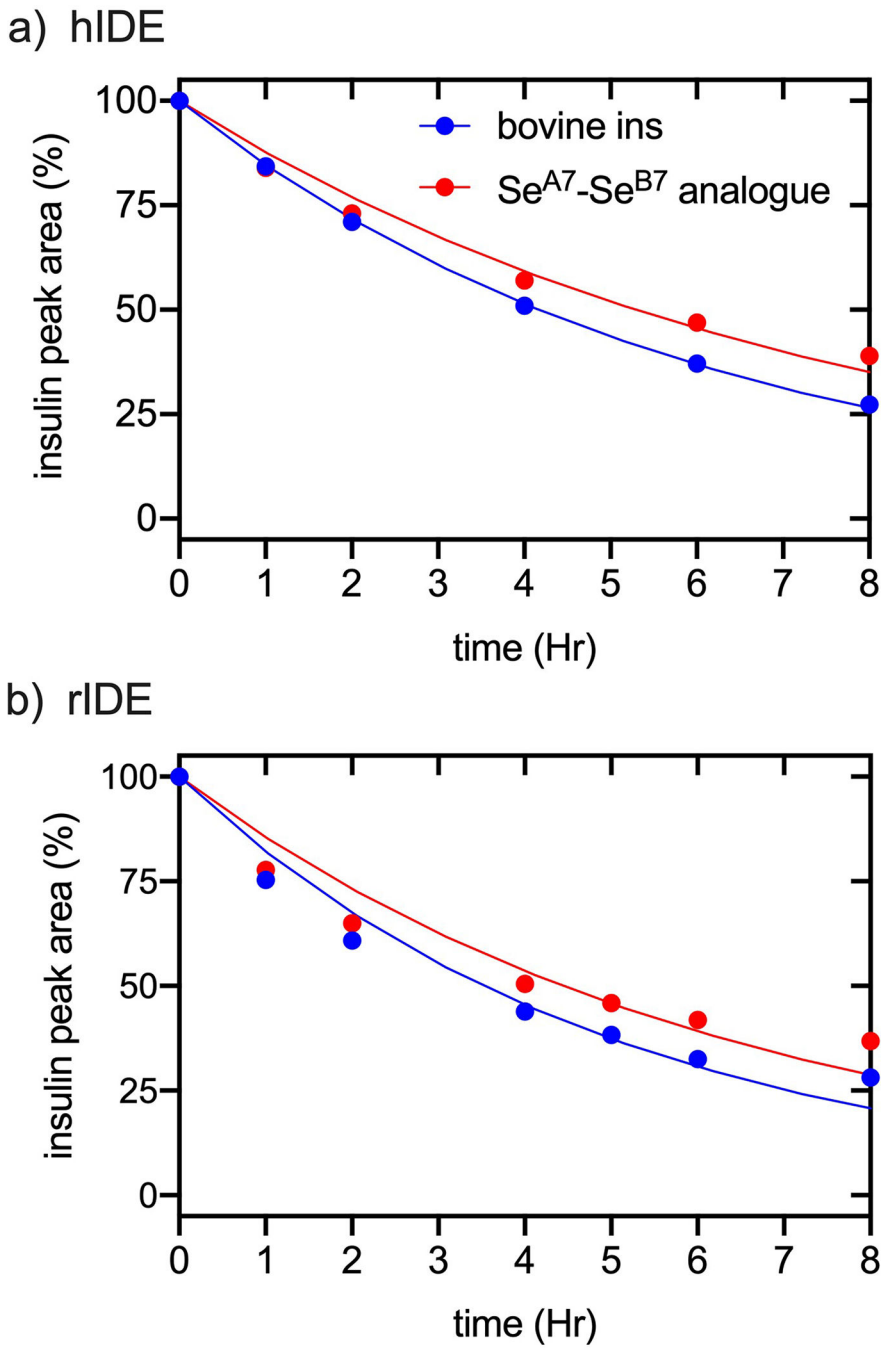


Figure 3.

Stability assays using insulin-degrading enzyme (IDE). **a**. Studies of human IDE. **b**. Studies of rat IDE; color code as in panel a Quantitative and statistical analyses are provided in SI Fig. S3.

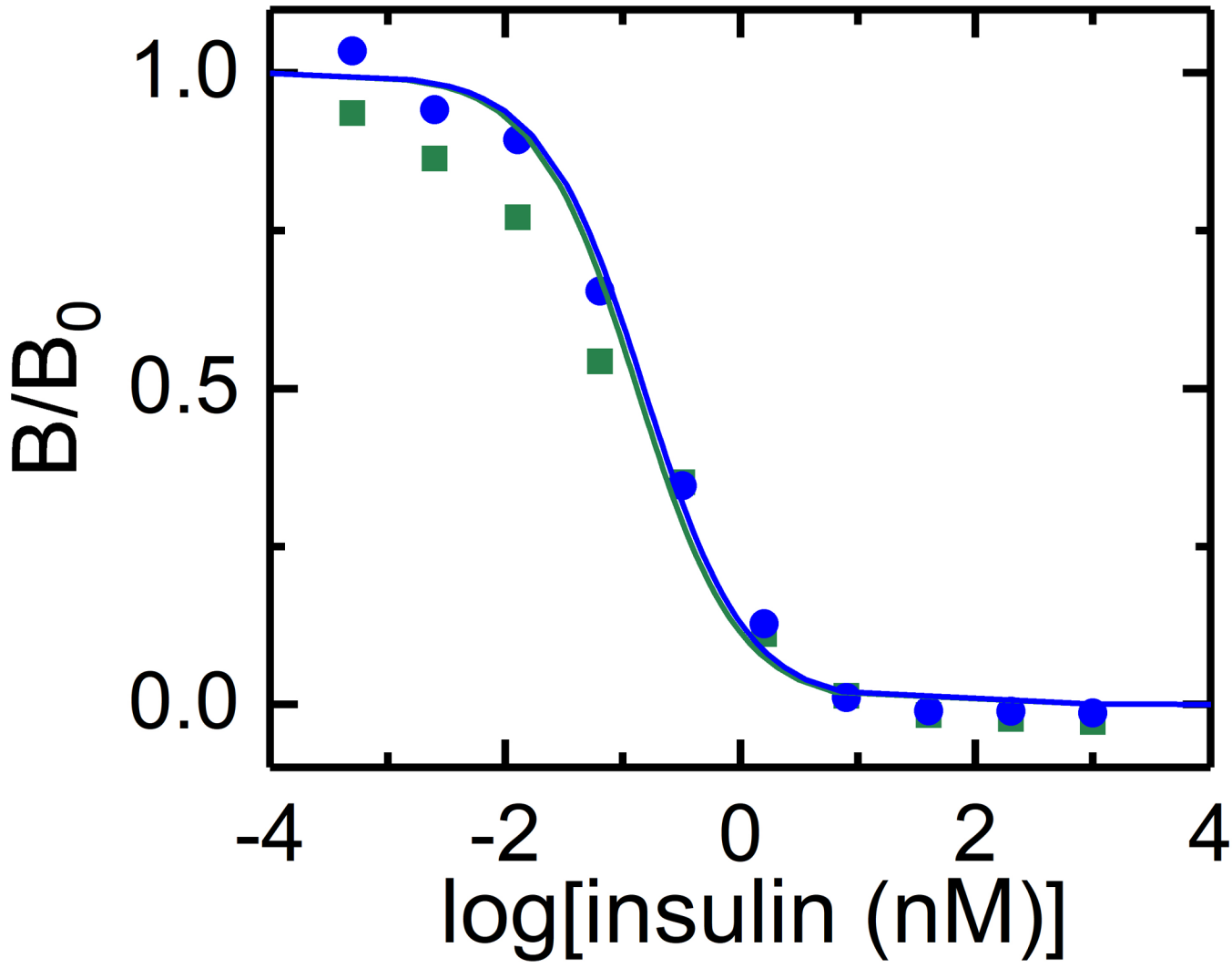


Figure 4.

Receptor-binding assay for insulin receptor (IR)[22b] for the Se-insulin analogue (●) compared to bovine ins (■). The two proteins present a similar binding affinity to IR with K_d = 130 ± 20 pM and 110 ± 10 pM respectively.

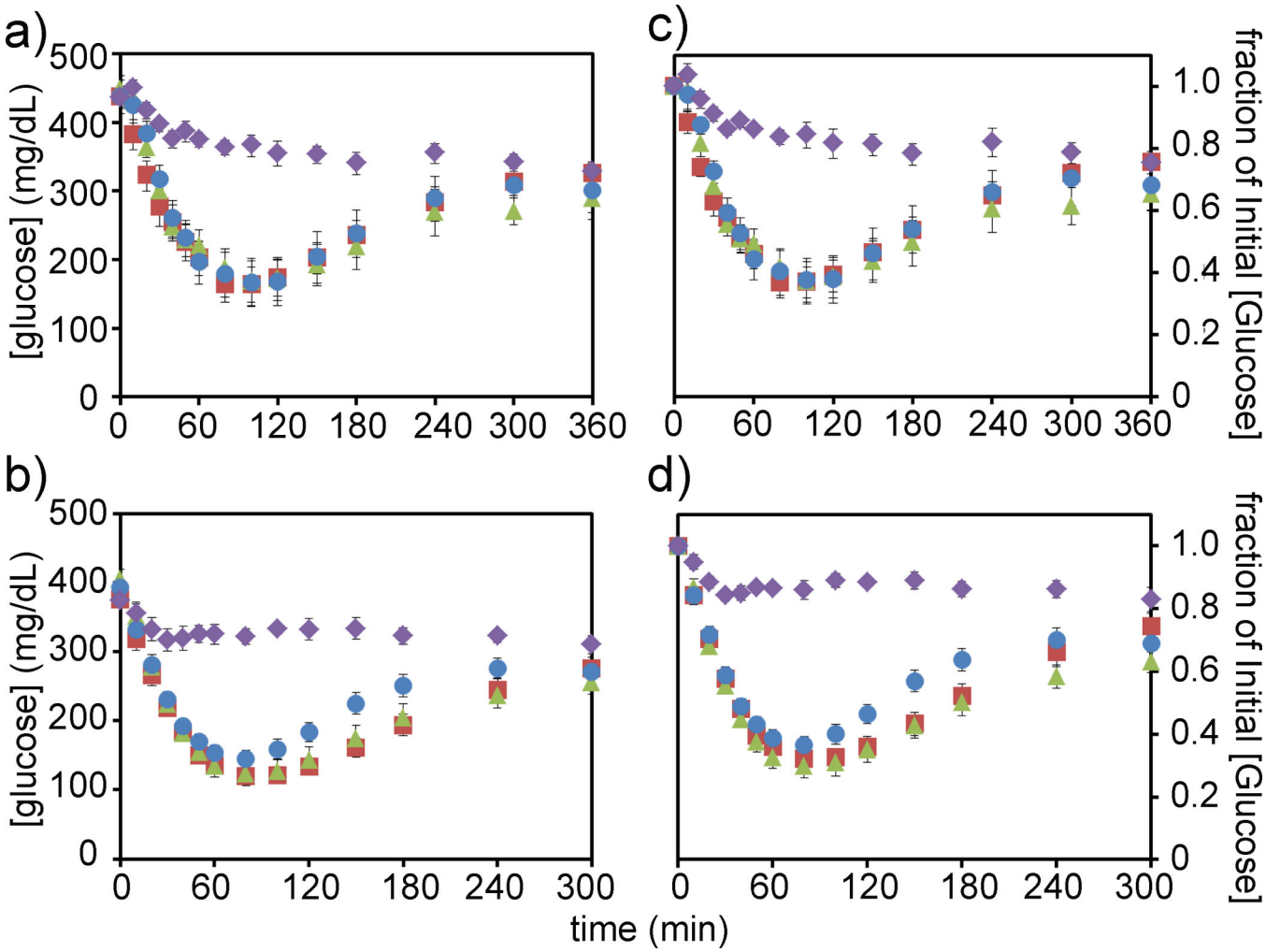
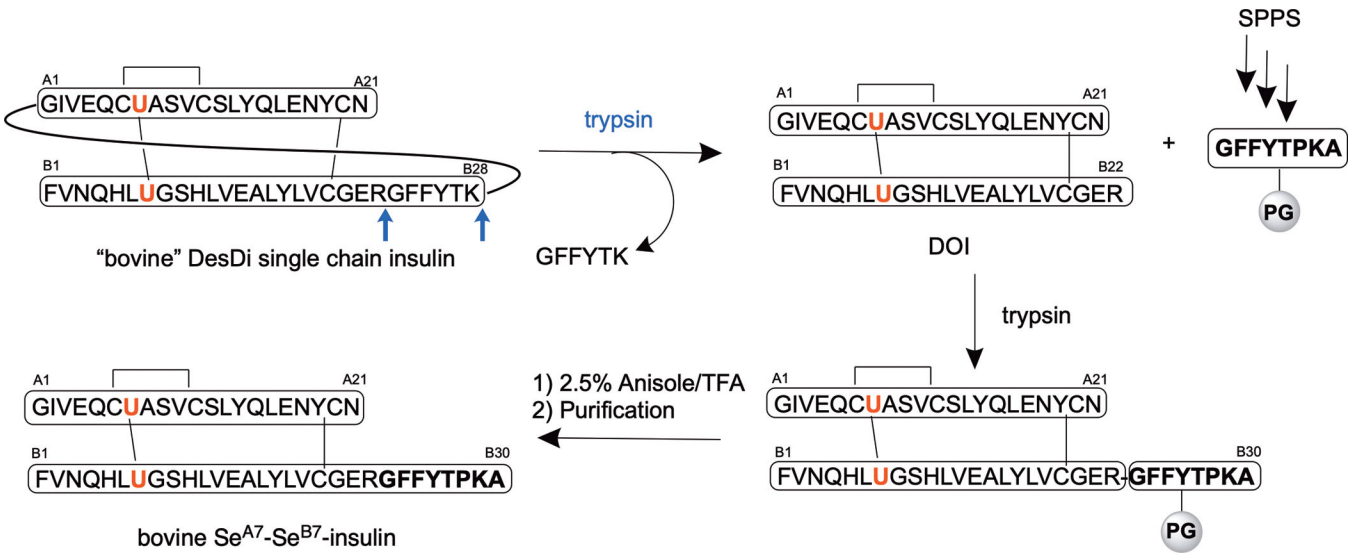


Figure 5.

Rat studies of insulin analogues. **a**. time course of [blood glucose] following SQ injection. Insulin lispro (■, n=15); bovine ins (▲, n=6); bovine Se-ins (●, n=6); diluent (◆, 100 μL, n=6). The dose of WT or modified insulin was 15 μg per 300-gram rat. **b**. time course of [blood glucose] following IV injection. Insulin lispro (n=20); bovine ins (n=12); bovine Se-ins (n=12); diluent (100 μL, n=5). This study used 10 μg insulin per 300-gram rat. (**c**) and (**d**), normalized blood glucose profiles for the data in panels **a** and **b**, respectively. Quantitative and statistical analyses are provided in SI Fig. S10–S12.



Scheme 1.

Synthetic strategy for making bovine Se-insulin[C7U^{A}, C7U^{B}] analogue. The control bovine WT insulin was synthesized adapting a similar strategy. DesDi represents Lys^{B28}-des-[B29,B30]-insulin; DOI – des-octapeptide[B23-B30]-bovine insulin, TFA – Trifluoroacetic acid.

Table 1.

Stabilities and protease sensitivity of insulin analogues.

analogue	ΔG_u[a] (kcal mol^{-1})	C_{mid}[a] (M)	m[b] (kcal mol^{-1} M^{-1})	degradation[c] $t_{½}$ (hr)		
				hIDE	rIDE	endo Glu-C
Insulin (b)	2.9 ± 0.1	4.7 ± 0.1	0.61 ± 0.01	4.17 ± 0.04	3.52 ± 0.23	0.49 ± 0.02
Se-insulin	2.8 ± 0.1	4.7 ± 0.1	0.58 ± 0.02	5.29 ± 0.26	4.44 ± 0.35	0.55 ± 0.04

[a] Parameters were inferred from CD-detected guanidine denaturation data (Fig. 2b) by application of a two-state model; uncertainties represent fitting errors for a given data set.

[b] The *m*-value (slope Δ(*G*)/Δ(M)) correlates with extent of hydrophobic surfaces exposed on denaturation.

[c] The $t_{½}$ values for IDE and V8 protease degradation (Fig. 3 and SI Fig. S5 respectively) of bovine insulin and Se-bovine insulin[C7U^{A}, C7U^{B}] were calculated based on a first-order decay function. IDE orthologues derived from human (hIDE) or rat (rIDE); endo Glu-C derived from *Staphylococcus aureus* V8.