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Thermodynamics of Formation of the Insulin Hexamer: Metal-Stabilized Proton-Coupled Assembly of Quaternary Structure

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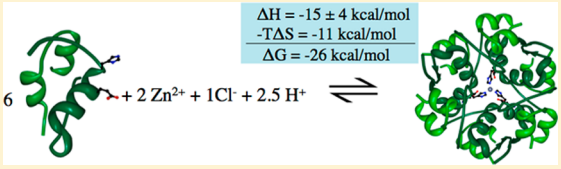
Supporting Information

ABSTRACT: The thermodynamics of formation of the insulin hexamer, which is stabilized by two Zn^{2+} ions, were quantified by isothermal titration calorimetry (ITC). Because the insulin monomer is unstable to aggregation (fibrillation) during ITC measurements, an original method involving EDTA chelation of Zn^{2+} from the hexamer was employed. The two metal ions are chelated sequentially, reflecting stepwise Zn^{2+} binding and stabilization of the quaternary structure. Analysis of the ITC data reveals that two to three H^+ bind to the hexamer upon its formation at pH 7.4, which is both enthalpically and entropically favored. The former is due to Zn^{2+} coordination to His residues from three subunits, and the latter is associated with desolvation that accompanies the protonation and the packing of the subunits in the hexamer.



In many cases, the biologically active form of a protein requires a complex consisting of two or more of the protein's subunits. The most thoroughly studied example of this quaternary structure is hemoglobin, which consists of a $\alpha_2\beta_2$ tetramer, where allosteric interactions between subunits are essential for the efficient delivery of O_2 from the lungs to the tissue. Although our understanding of the forces and interactions that stabilize unique protein structures has advanced considerably, the molecular interactions that stabilize discrete dimers, trimers or tetramers, and not extended oligomers, of a protein are not as well understood. Because these interactions need to overcome the entropic penalty for assembly of the complex, there is a need to quantify the thermodynamics of quaternary-structure formation.

In some cases, protein quaternary structure is stabilized by metal ions. This can involve the electrostatic interactions of metal ions at a protein−protein interface[1] or metal coordination resulting in a protein conformation that is competent for dimerization, with Ca^{2+} binding to EF hand domains being a common example.[2] Metals can also stabilize quaternary structure through bridging coordination, which is seen in Zn^{2+} stabilization of an active dimer of a number of enzymes.[3−5] Metal coordination has also been used in the formation of supramolecular structures, including those constructed with protein subunits.[6]

One of the earliest recognized examples of metal-stabilized quaternary structure is the hexamer of insulin. Synthesized in the β cells of the pancreas, where it is cleaved into two disulfide-linked peptide chains (21 residue A and 30 residue B), insulin is stored in Zn^{2+}- and Ca^{2+}-rich vesicles as a hexamer that, upon release into the bloodstream, dissociates into monomers that bind the insulin receptor to regulate blood-glucose levels.[7] The monomer, however, is susceptible to fibrillation,[8,9] but the hexamer is not, making it the preferred form in pharmaceutical formulations for the treatment of diabetes.

The insulin hexamer has been structurally well-characterized by X-ray crystallography (Figure 1).[10,11] It is stabilized by two

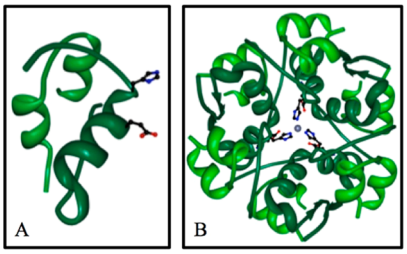


Figure 1. (A) Insulin monomer with B10His and B13Glu highlighted. (B) T_6 bovine insulin hexamer viewed down its C_3 symmetry axis (PDB: 2A3G).

Zn^{2+} ions each coordinated to three B10 histidines from three of the subunits. Depending on the presence of allosteric effectors, insulin monomers in the hexamer adopt one of two conformations: *T*, with its B1−9 residues in an elongated conformation, or *R*, with its B1−9 residues in a α helix, which exposes a hydrophobic phenol-binding pocket. Three forms of the hexamer are stabilized by coordinating anions (e.g., Cl^-) and phenols: T_6 (Zn^{2+}), T_3R_3 (Zn^{2+}, Cl^-), and R_6 (Zn^{2+}, Cl^-, phenol). The Zn^{2+} coordination correlates with the subunit conformations and has three additional waters when the three subunits are in the *T* conformation but has a single coordinating anion when the three subunits are in the *R* conformation. The structure of the hexamer orients the B13 glutamic acids of the six subunits toward the center, which

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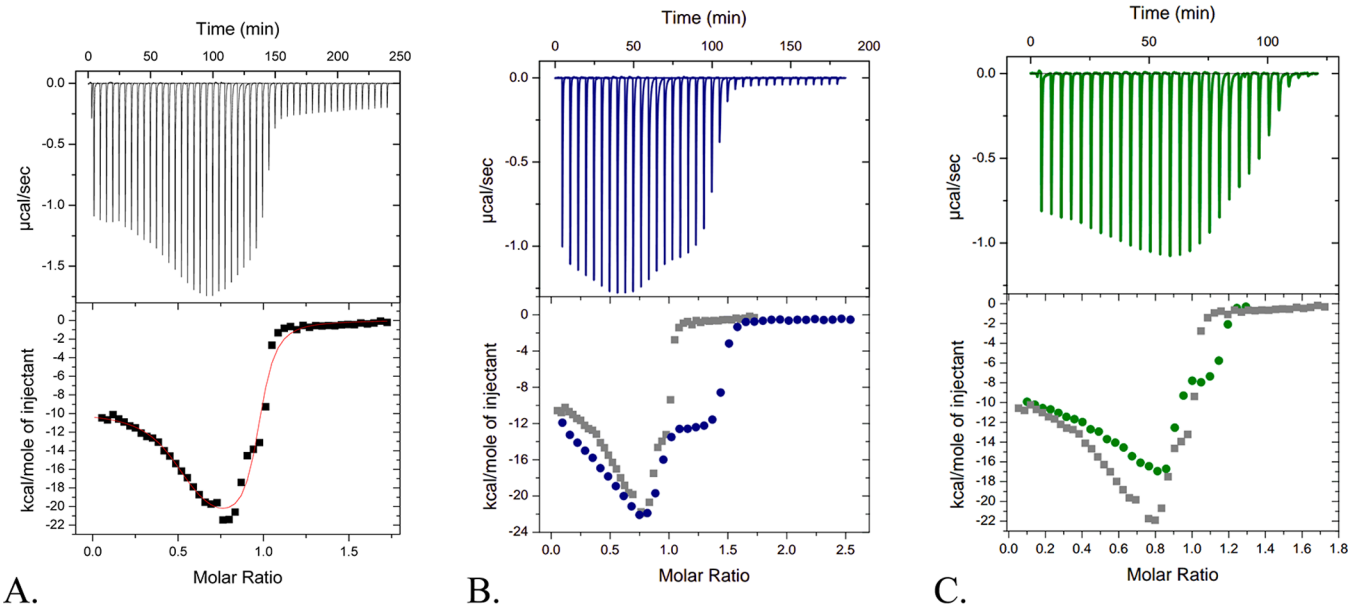

Figure 2. Representative ITC thermograms of EDTA chelation of insulin hexamers. (A) T_3R_3; 0.38 mM EDTA → 0.05 mM $ZnCl_2$ + 0.15 mM human insulin monomer; fit to two-sites model (lower panel): n_1= 0.50 ± 0.03, K_1 = 7 (±5) × 10^7, ΔH_1 = −9.8 ± 0.8 kcal mol^{-1}, n_2 = 0.46 ± 0.03, K_2 = 4 (±1) × 10^6, ΔH_2 = −23 ± 1 kcal mol^{-1}. (B) T_3R_3'; 0.28 mM EDTA → 0.027 mM $ZnCl_2$ + 0.013 mM $CaCl_2$ + 0.08 mM human insulin monomer; lower panel compares the integrated, concentration-normalized T_3R_3' data (blue) to that of T_3R_3 (gray) from panel A. (C) T_6; 0.28 mM EDTA → 0.027 mM $ZnSO_4$ + 0.08 mM human insulin monomer; lower panel compares the integrated, concentration-normalized T_6 data (green) to that of T_3R_3 (gray) from panel A. All measurements were obtained in 50 mM Tris buffer, pH 7.4.

creates a cation-binding cavity where one Ca^{2+} or >2 Na^+ and a water network are found.[11,12]

Early studies used equilibrium dialysis to quantify Zn^{2+} binding to insulin.[13−15] More recently, Dunn and co-workers have used Co^{2+} and Cd^{2+} as surrogates for Zn^{2+} and Ca^{2+} in a series of spectroscopic and kinetic studies of hexamer formation,[16] and Brader has used the Zn-binding chromophore zincon to characterize allosteric interactions among the insulin subunits along the $T_6 \rightarrow T_3R_3 \rightarrow R_6$ pathway.[17] Differential scanning calorimetry (DSC) has been used to investigate the thermal stability of the insulin hexamer,[18−20] and isothermal titration calorimetry (ITC) has been used to quantify the thermodynamics of phenols binding to the hexmer, which is complex because of interactions among the subunits.[21] However, other than a study of porcine insulin that used unbuffered flow calorimetry,[22] the thermodynamics of insulin hexamer formation have not been reported. Here, we have used ITC to quantify Zn^{2+} binding to human insulin and to determine the thermodynamics of formation of its hexamer quaternary structure.

EXPERIMENTAL SECTION

Human insulin was purchased from Sigma-Aldrich and prepared as follows. Five milligrams of insulin was dissolved in 2 mL of nanopure (18 MΩ) water, and the pH was adjusted to ∼3. This solution was applied to a 28 × 0.5 cm Chelex 100 column, and the collected protein was transferred to the buffer solution using a 3500 Amicon centrifuge filter. The insulin concentration was determined by A^{276} using ε = 6200 $M^{-1}cm^{-1}$,[23] and its identity and purity were confirmed by a single 5.8 kDa peak in the MALDI-MS. The appropriate concentration(s) of metals required for the desired hexamer form were added immediately to the protein. Preliminary studies were done with porcine insulin, also obtained from Sigma-Aldrich and prepared as described, which differs from human insulin only by the B30 residue (Thr in human vs Ala in porcine) and gives very similar results. The insulin monomer is in equilibrium with its dimer,[24] which is the dominant form under our experimental conditions.

ITC measurements were obtained at 25 ± 0.2 °C with a MicroCal VP-ITC stirring at 300 rpm. The data were fit with manufacturer-supplied models running in Origin 7. The data are presented as the raw experimental data (heat flow per second vs time) with a flattened baseline in the top panel and the integrated concentration-normalized data (heat flow per mole of injectant vs binding stoichiometry) in the lower panel.

RESULTS AND ANALYSIS

Initial ITC measurements involved titrating Zn^{2+} into solutions of insulin, but these gave frustratingly inconsistent binding stoichiometries and enthalpies. The aggregation and subsequent fibrillation of insulin can be accelerated by high ionic strength, high and low pH, high temperature, high insulin concentrations, high Zn^{2+} concentrations, and agitation[25] and can be followed with several methods, including circular dichroism (CD), microscopy, and fluorescence.[26,27] Because titration calorimetry requires stirring to achieve rapid equilibrium after the addition of each aliquot, we suspected that aggregation (fibrillation) of the insulin was complicating the calorimetric measurements. This was confirmed by CD (Figure S1), microscopy (Figure S2), and DSC (Figure S3) characterization of insulin samples before and after the calorimetric titrations.

To avoid aggregation (fibrillation) during ITC measurements, ethylenediaminetetraacetic acid (EDTA) was titrated into solutions of the Zn_2Ins_6 hexamer, which is resistant to fibrillation. This gave reproducible thermograms with two inflections associated with EDTA chelation of the two Zn^{2+} ions, indicating that they are bound with different affinities and enthalpies (Figure 2A).

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A two-sites binding model provides a good fit of the sequential chelation of Zn^{2+} from the hexamer (Scheme 1).

Scheme 1. Sequential EDTA Chelation of Zn^{2+} from the Insulin Hexamer

$$EDTA + Zn_2Ins_6 \rightleftarrows ZnEDTA + [ZnIns_4] + Ins_2$$

$$EDTA + [ZnIns_4] \rightleftarrows ZnEDTA + 2\ Ins_2$$

Insulin samples removed from the calorimeter after these measurements have DSC thermograms showing that, in contrast to direct Zn^{2+} titrations (Figure S3), insulin maintains its tertiary protein structure over the course of the chelation titration (Figure S4).

In addition to T_3R_3 (Figure 2A), chelation ITC measurements were made on T_3R_3 with one Ca^{2+}, designated T_3R_3' (Figure 2B). For these samples, the first two chelation events are quantitatively similar to those of T_3R_3 (Figure S5), indicating that the Ca^{2+} does not significantly affect the Zn^{2+} chelation thermodynamics. However, a third binding event is observed and ascribed to EDTA chelation of the Ca^{2+} subsequent to chelation of the two Zn^{2+} ions. Equilibrium dialysis has shown that Ca^{2+} binds to the Zn_2Ins_6 hexamer with $K = 1.2 \times 10^4$.[12] Because this value is smaller than Zn^{2+} stability constants that have been reported previously[13−15] and that we have determined with ITC (vide infra), these results indicate that there is a significant kinetic barrier to Ca^{2+} chelation from the B13Glu cavity until both Zn^{2+} ions have been chelated by EDTA.

Chelation ITC measurements have also been made on T_6 (Figure 2C), and these are qualitatively similar to those of T_3R_3 except for the enthalpy of the second chelation event, which is quantitatively smaller (Figure S6). This indicates that the first Zn^{2+} removed from T_3R_3 by EDTA is the one that is coordinated to the three T subunits in an $\sim O_h$ geometry with three waters. The different enthalpy for the second chelation event indicates that coordinating anions (e.g., Cl^-) affect the enthalpy of formation of the intermediate species with a single Zn^{2+}.

The T_3R_3 form was chosen for quantitative analysis of the thermodynamics of hexamer formation. Initially, it was necessary to determine if there were any enthalpic contributions from (de)protonations coupled with Zn^{2+} binding and hexamer formation. To do so, the experimental chelation enthalpies (ΔH_{ITC}) were measured in three different buffers at pH 7.4 (Table 1, columns a and b).

Analysis of these data is based on the assumption that removal of Zn^{2+} from the hexamer is the microscopic reverse of Zn^{2+} binding and stabilizing the insulin hexamer. Therefore, thermodynamic values determined from the chelation measurements (Scheme 1), after accounting for the EDTA-Zn^{2+} interaction, are equal in magnitude but opposite in sign to those for binding and formation (Scheme 2).

Scheme 2. Sequential Formation of the Zn-Stabilized Insulin Hexamer

$$Zn^{2+} + 2\ Ins_2 \rightleftarrows [ZnIns_4]$$

$$Zn^{2+} + [ZnIns_4] + Ins_2 \rightleftarrows Zn_2Ins_6$$

Using the assumption of microscopic reversibility and subtracting the enthalpy associated with the EDTA chelation (Table 1, column c) gives the enthalpies for the two sequential Zn^{2+} binding steps and the total enthalpy of formation of the insulin hexamer in the different buffers (Table 1, columns d−f). A plot of these values against the buffer protonation enthalpy (Table 1, column g) allows the number of protons involved in each step of hexamer formation to be determined[29] (Figure S7). This analysis reveals that 3 ± 1 protons are bound to the protein as the first Zn^{2+} binds and 0.3 ± 0.6 protons are released as the second Zn^{2+} binds, giving a total of 2.5 ± 0.4 protons bound to the hexamer upon its formation. These values are then used to account for the contributions of buffer (de)protonation enthalpy to the experimental enthalpy and finally to calculate the buffer-independent enthalpies for the two steps and the total enthalpy of formation of the insulin hexamer (Table 1, columns h−j). The average buffer-independent enthalpies are −18 ± 2 kcal/mol for the first step, 2 ± 3 kcal/mol for the second step, and −15 ± 4 kcal/mol for the overall hexamer formation.

Chelation ITC measurements also allow the stability constants, and therefore ΔG values, for the two-step formation of the T_3R_3 hexamer to be determined. Although the experimental data can be fit to a two-sites model to determine the binding enthalpies (Figure 2A), the titration involves a competition between EDTA and insulin for Zn^{2+} and there is a significant change in the concentration (activity) of free insulin. However, by using a competition binding model[30] and the known thermodynamics of the EDTA−Zn^{2+} interaction, the stability constants can be determined.

Because the second chelation step is significantly more exothermic than the first, it is not possible to determine an accurate stability constant for the first chelation step (second Zn^{2+} binding) with this model. However, the well-resolved inflection of the second chelation step allows the stability constant for this step (first Zn^{2+} binding) to be determined

Table 1. Chelation ITC Enthalpies for T_3R_3 Insulin Hexamer and Analysis to Obtain Buffer-Independent Enthalpies of Formation at 25°C, pH 7.4[a]

	experimental ITC chelation values		→	buffer-dependent Zn^{2+} binding			→	buffer-independent Zn^{2+} binding		
	a	b	c	d	e	f	g	h	i	j
				(−b + c)	(−a + c)	(d + e)		(2.9g + d)	(−0.3g + e)	(2.5g + f)
buffer	ΔH_{ITC1}	ΔH_{ITC2}	$\Delta H_{EDTA\text{-}Zn}$[b]	ΔH_{Zn1}	ΔH_{Zn2}	$\Delta H_{Zn(1+2)}$	ΔH_{HBuff}[c]	ΔH_{Zn1Ins}	ΔH_{Zn2Ins}	$\Delta H_{Zn(1+2)Ins}$
Tris	−9 ± 1	−28 ± 4	−11.2	17	−2	15	−11.4	−17 ± 4	1 ± 4	−14 ± 6
Bis-Tris	−6 ± 2	−10 ± 3	−6.5	3	−0.5	3	−6.9	−17 ± 2	1 ± 2	−14 ± 3
ACES	−10 ± 6	−16 ± 5	−9.1	7	1	8	−9.3	−21 ± 7	4 ± 6	−16 ± 9

[a]Units of kcal/mol. [b]$\Delta H_{EDTA\text{-}Zn} = -1.05\Delta H_{HEDTA} + \Delta H_{ZnEDTA} + 1.05\Delta H_{HBuff}$. [c]Ref 28.

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(Figure S8) with some accuracy as $(2.0 \pm 0.9) \times 10^{10}$, giving $\Delta G = -14.0 \pm 0.4$ kcal/mol for the first step in the formation of the insulin hexamer. Because the free energy of the second formation step (first chelation step) cannot be determined with this competition model, the fit of the data with the two-sites model was used to estimate the magnitude of this stability constant relative to that of the first formation step. These values consistently differ by 1 order of magnitude, so the stability constant for the second step in the formation of the hexamer is estimated to be $\sim 2 \times 10^9$, giving a free energy of about −12 kcal/mol. Table 2 summarizes the thermodynamics of formation of the T_3R_3 insulin hexamer.

Table 2. Thermodynamics of T_3R_3 Insulin Hexamer Formation at 25°C, pH 7.4

	number of H^+	ΔH^a	$-T\Delta S^a$	ΔG^a
first Zn^{2+}	+3 ± 1	−18 ± 2	4 ± 2	−14.0 ± 0.4
second Zn^{2+}	−0.3 ± 0.6	2 ± 3	−14	−12
Zn_2Ins_6	+2.5 ± 0.4	−15 ± 4	−11	−26

[a]Units of kcal/mol.

DISCUSSION

The insulin binding constants for Zn^{2+} that were determined earlier with equilibrium dialysis are significantly smaller (10^5 to 10^6)[13−15] than those found here. Furthermore, the stoichiometry reported earlier varies from 0.3 to 1.0 Zn^{2+} per monomer, followed by a second Zn^{2+} binding event with a lower stability constant. Only recently has the propensity of insulin to aggregate and form fibrils been well-characterized[25−27] and is better understood.[31] In our direct ITC measurements, where we find evidence for aggregation (fibrillation) of the insulin, we also find a stoichiometry that ranges from 0.3 to >1, as well as a second Zn^{2+} binding event (Figure S3). This suggests that aggregation (fibrillation) may have compromised earlier measurements of Zn^{2+} binding to insulin.

The thermodynamic data in Table 2 provide new insight about the two-step formation of the T_3R_3 insulin hexamer. The first step, which involves one Zn^{2+}, at least two insulin dimers, and approximately three protons, is enthalpically driven. Because the enthalpy of each Zn^{2+}−His bond is estimated to be about −5 kcal/mol,[32] coordination to three histidines would contribute about −15 kcal/mol. If the first Zn^{2+} brings the B13 glutamates of four or more insulin monomers of this intermediate into close proximity, then the incipient cation-binding cavity could be the binding site for the three protons, which would also contribute to the binding enthalpy. Somewhat surprisingly, the net entropy change for this step is small. The expected entropic penalty for assembly of the intermediate would be reduced by desolvation of the three protons and the Zn^{2+} upon binding to the protein.

The second step is entropically driven, with negligible net change in enthalpy. The exothermic contribution from the formation of three new Zn^{2+}−His bonds must be canceled by endothermic contributions, possibly from the three subunits that now adopt the R conformation. The favorable change in entropy of this step is attributed to desolvation of the second Zn^{2+} as well as further desolvation of the insulin subunits as they lock into the compact hexamer structure, which has fewer bound waters than other proteins on a per-residue basis.[10]

The thermodynamics of Zn^{2+} stabilization of the insulin T_3R_3 quaternary structure is shown here to be both enthalpically and entropically favored, as was found for Zn^{2+} stabilizing the secondary and tertiary structure of zinc fingers.[33] Because two Zn^{2+} ions each binding to three histidines would be expected to contribute about −30 kcal/mol to the binding enthalpy, endothermic contributions, possibly associated with the $T \rightarrow R$ conformational change of three subunits, reduce this considerably. Although an entropic penalty is expected for hexamer assembly, this is canceled, apparently by a significant amount of desolvation from the two bound Zn^{2+} ions, the 2.5 bound protons, and efficient packing of the six insulin subunits in the hexamer structure.

The insulin quaternary structure is uniquely tuned for its biological role. The hexamer suppresses degradation and fibrillation during storage in the pancreas, but it must dissociate upon release into the bloodstream so that the insulin monomer can bind to its receptor. A metal-stabilized complex (Figure 1B) allows for both of these properties. Zinc coordination to the B10His residues provides discrete contacts between the subunits and stabilizes them in a specific structure, in this case a hexamer (trimer of dimers) with a cation-binding cavity in the center. However, dissociation of the hexamer upon its release in the bloodstream is favored by the low level of free Zn^{2+} because of the presence of chelating proteins, such as serum albumin ($\log K_{Zn} = 7.3$).[34]

The discovery that two-to-three H^+ bind to the hexamer upon its formation indicates that the quaternary structure increases one or more pK_a's. A similar phenomenon has been observed for hemoglobin, where the pH-dependence of the equilibrium between the $\alpha\beta$ dimer and the $\alpha_2\beta_2$ tetramer was used to determine that 0.9 H^+ bind upon formation of the deoxy tetramer and 0.8 H^+ are released upon formation of oxy tetramer.[35] These results were then used to determine the Bohr effect of the dimer for comparison to that of the tetramer.[36,37] In the case of insulin, protons that bind to the hexamer upon its formation appear to contribute to desolvation that counters the entropic penalty of its formation. This also creates a pH-dependent switch that helps to destabilize the insulin hexamer when it is released from β-cell vesicles (pH ∼ 5.5) into the bloodstream (pH 7.4).[7]

In summary, this appears to be the first quantitative analysis of the thermodynamics of assembly of a quaternary structure. Formation of the insulin hexamer is enthalpically driven by Zn^{2+} coordination to the histidines, yet the assembly is also entropically favored, which is attributed to desolvation associated with protonation of the subunits and their efficient packing in the hexamer. Modulation of pK_a's may be a useful strategy to provide an entropic compensation for the assembly of quaternary structure. Ongoing studies will quantify the thermodynamic contributions of phenols to the formation of the R_6 hexamer.

ASSOCIATED CONTENT

Supporting Information

Calorimetric (ITC, DSC) data and analysis of ITC data, spectroscopic (CD) data, and microscopy on insulin samples, as well as proton analysis from experimental enthalpies. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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