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Research Paper

Chemical and Thermal Stability of Insulin: Effects of Zinc and Ligand Binding to the Insulin Zinc-Hexamer

Kasper Huus,[1,2,3] Svend Havelund,[2] Helle B. Olsen,[2] Marco van de Weert,[1] and Sven Frokjaer[1]

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Purpose. To study the correlation between the thermal and chemical stability of insulin formulations with various insulin hexamer ligands.
Materials and Methods. The thermal stability was investigated using differential scanning calorimetry (DSC) and near-UV circular dichroism (NUV-CD). The formation of chemical degradation products was studied with reversed-phase and size-exclusion chromatography and mass spectrometry.
Results. An excellent correlation between the thermal stabilization by ligand binding and the deamidation of Asn^{B3} was observed. The correlation between thermal stability and the formation of covalent dimer and other insulin related products was less clear. Zinc was found to specifically increase the deamidation and covalent dimer formation rate when the insulin hexamer was not further stabilized by phenolic ligand. Thiocyanate alone had no effect on the thermal stability of the insulin zinc-hexamer but significantly improved the chemical stability at 37°C. At low temperatures thiocyanate induced a conformational change in the insulin hexamer. NUV-CD thermal scans revealed that this effect decreased with temperature; when the thermal denaturation temperature was reached, the effect was eliminated.
Conclusions. Thermal stability can be used to predict the rate of Asn^{B3} deamidation in human insulin. Chemical degradation processes that do not rely on the structural stability of the protein do not necessarily correlate to the thermal stability.

KEY WORDS: chemical stability; deamidation; differential scanning calorimetry; dimerization; insulin; protein stability; thermal stability.

INTRODUCTION

Insulin was introduced as a drug in the 1920s. Since then, extensive work has been done, and is still ongoing, to adjust the pharmacokinetics and improve the stability of formulations of insulin and insulin analogues. Numerous studies have been published relating especially to the chemical stability and the fibrillation of insulin (1–3). The primary object of the present work is to evaluate the use of differential scanning calorimetry (DSC) as a method to predict the chemical stability of insulin formulations. The rapidity of DSC compared to traditional storage tests would make it extremely valuable in early formulation screening.

Insulin interacts with insulin receptors as a monomer, but in pharmaceutical formulations, insulin is generally present as hexamers in coordination with zinc (4). We have previously studied the effect of zinc concentration on the thermal denaturation of insulin (5). At zinc concentrations typically used in soluble insulin formulations (around 3 Zn^{2+}/hexamer) insulin exhibited a biphasic denaturation, where both a dimer and a hexamer denaturation transition were observed. At higher zinc concentrations (5 Zn^{2+}/hexamer or more) only the hexamer denaturation transition was observed (5). In a DSC study of the binding of phenolic and anionic ligands to the insulin hexamer we used 5 Zn^{2+}/hexamer in order to exclusively study the stability of the hexamer without disturbance from the dimer–hexamer equilibrium. The ligand-induced increases in thermal stability measured by DSC matched the affinity of the ligands to the insulin hexamer found by other methods (6).

Differential scanning calorimetry is increasingly used in the stability screening of biopharmaceutical liquid formulations. In general, the thermal transition midpoint (T_m) from DSC experiments is expected to be related to degradation processes that depend on the tendency of the protein to unfold (7). A number of studies have found correlation between the ranking of the physical stability of formulations

[1] Department of Pharmaceutics and Analytical Chemistry, The Danish University of Pharmaceutical Sciences, 2100 Copenhagen, Denmark.
[2] Diabetes Protein Engineering, Novo Nordisk A/S, 2880 Bagsvaerd, Denmark.
[3] To whom correspondence should be addressed. (e-mail: ksan@novonordisk.com)

ABBREVIATIONS: CD, circular dichroism; C_p, excess heat capacity; DSC, differential scanning calorimetry; MALDI-TOF, matrix-assisted laser desorption ionization mass spectrometry; PABA, *para*-aminobenzoic acid ; RP-HPLC, reversed-phase high-performance liquid chromatography; SEC, size-exclusion chromatography; T_m, transition midpoint; 4H3N, 4-hydroxy-3-nitrobenzoic acid.

measured as thermal stability by DSC and physical stability measured by other methods (8–12). This correlation is expected, because DSC and agitation tests both relate to the folding stability of the protein. The correlation between DSC results and long term chemical stability of protein formulations is not straightforward, because many factors other than the overall stability against unfolding play a role in the chemical degradation of proteins (7,13). However, chemical degradation processes (such as deamidation) that occur in sites of the protein, which are protected in the native state, proceed faster when the protein is unfolded (14–17). In a study of different monomeric insulin mutants, correlation was found between the conformational stability (determined by guanidine hydrochloride-induced denaturation) and the rate of covalent polymerization (18). When chemical degradation processes correlate to the conformational stability of the protein, they will most likely also relate to the thermal stability. On the other hand, chemical degradation processes that take place for example near the surface of the protein are not necessarily expected to increase in rate when the protein is unfolded. Consequently, formulation influences on such degradation processes are not likely to be revealed by DSC studies.

In the present work we will evaluate the use of DSC in the studies of insulin stability when zinc is present at formulation-relevant concentrations and examine the correlation with the chemical stability of insulin formulations. Furthermore we seek to examine the effect of thiocyanate in the absence of phenolic ligands. We have previously found that with 5 Zn^{2+}/hexamer, thiocyanate did not influence the thermal denaturation of insulin although other studies have established that thiocyanate stabilizes the zinc-hexamer (6,19). In the present work we will clarify this discrepancy.

MATERIALS AND METHODS

Sample Preparation

Zinc-free native human insulin was obtained from Novo Nordisk A/S. All other chemicals were of analytical grade. Deionized water was filtered using a Millipore system (Millipore, Billerica, MA) and used for all samples. All insulin samples were prepared in 7 mM phosphate buffer adjusted to pH 7.4 with perchloric acid and/or sodium hydroxide. Zinc was added as either the acetate or chloride salt. All samples were made from fresh stock solutions to give a concentration of 0.6 mM insulin. The concentration of the stock solutions was determined from the absorbance at 276 nm using an ε_{276} of 6,200 M^{-1} cm^{-1} (20). The two ligands *p*-aminobenzoic acid and 4-hydroxy-3-nitrobenzoic acid are referred to by the abbreviations PABA and 4H3N, respectively.

Chemical Stability Assessment with Size-Exclusion and Reversed-Phase Chromatography

The insulin samples were filled in glass vials and stored at 37°C for 2 weeks. Controls were stored at 5°C. The content of soluble covalent di- and polymer products was analyzed by size-exclusion chromatography (SEC). Samples (40 μl) were subjected to SEC at room temperature using a Waters insulin HMWP column 7.8 × 300 mm (Waters Corporation, Milford, MA, USA) at a flow rate of 1 ml/min with an eluent comprising 15 volumes of glacial acetic acid, 20 volumes of acetonitrile and 65 volumes of a 1.0 g/l solution of arginine. Detection was performed at 276 nm. The content of deamidation products and other insulin related substances was analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC). The method is identical to the assay for purity and content, which has been described previously (21). All results are given as the amount of degradation product formed, where the content in control samples is subtracted from the content in the samples after two weeks at 37°C. The term "other insulin related substances" is used in the present work. This is the total amount of degradation products found by RP-HPLC except for the deamidation products and the di- and polymer products that were quantified by SEC.

MALDI-TOF Mass Spectrometry

SEC chromatography fractions were collected and evaporated under vacuum to approximately one half of the original volume. Fractions (3 μl each) corresponding to the species smaller than insulin monomer were applied to α-cyano-4-hydroxycinnamic acid prespotted anchor chip (PAC384, Bruker Daltonics, Bremen, Germany), blotted after approximately 30 s with tissue paper and the spots were washed twice with 3 μl of 15 mM ammonium phosphate solution. The spots were analyzed manually using an Autoflex TofTof mass spectrometer (Bruker Daltonics) in both reflector mode (monoisotopic mass resolution, m/z = 500−4,000, calibrated using a standard peptide mixture, Bruker Daltonics) and linear mode (average mass resolution, m/z = 3000−8000, calibrated using a home made calibration mixture). Typically, 100–200 laser shots were averaged for each spot.

Differential Scanning Calorimetry

Data collection was performed using a VP-DSC differential scanning calorimeter (MicroCal, LLC, Northampton, MA; 22). Temperature scans were performed from 25 to 110°C at a scan rate of 1°C/min and an excess pressure of 0.21 MPa with 7 mM phosphate buffer in the reference cell. The samples and references were degassed immediately before use. A buffer-buffer reference scan was subtracted from each sample scan prior to concentration normalization. Baselines were created in Origin 7.0 (OriginLab, Northampton, MA) by cubic interpolation of the pre- and post-transition baselines. T_{max} is the temperature at which the excess heat capacity, C_p, is at its maximum.

Near-UV Circular Dichroism

Near-UV circular dichroism (CD) spectra were obtained on a Jasco J-715 circular dichroism spectrophotometer (Jasco, Tokyo, Japan). The filtered insulin samples were scanned in a 0.1 or 0.2 cm cell from 320 to 250 nm using a bandwidth of 2.0 nm, 2 s response time, a data pitch of 0.5 nm and a scanning speed of 20 nm/min. A peltier element was used to control the heating of the CD samples during temperature scans. Each spectrum is the accumulation of two scans. Spectra of the buffer were recorded and subtracted from each sample spectrum. The protein concentration obtained by UV absor-

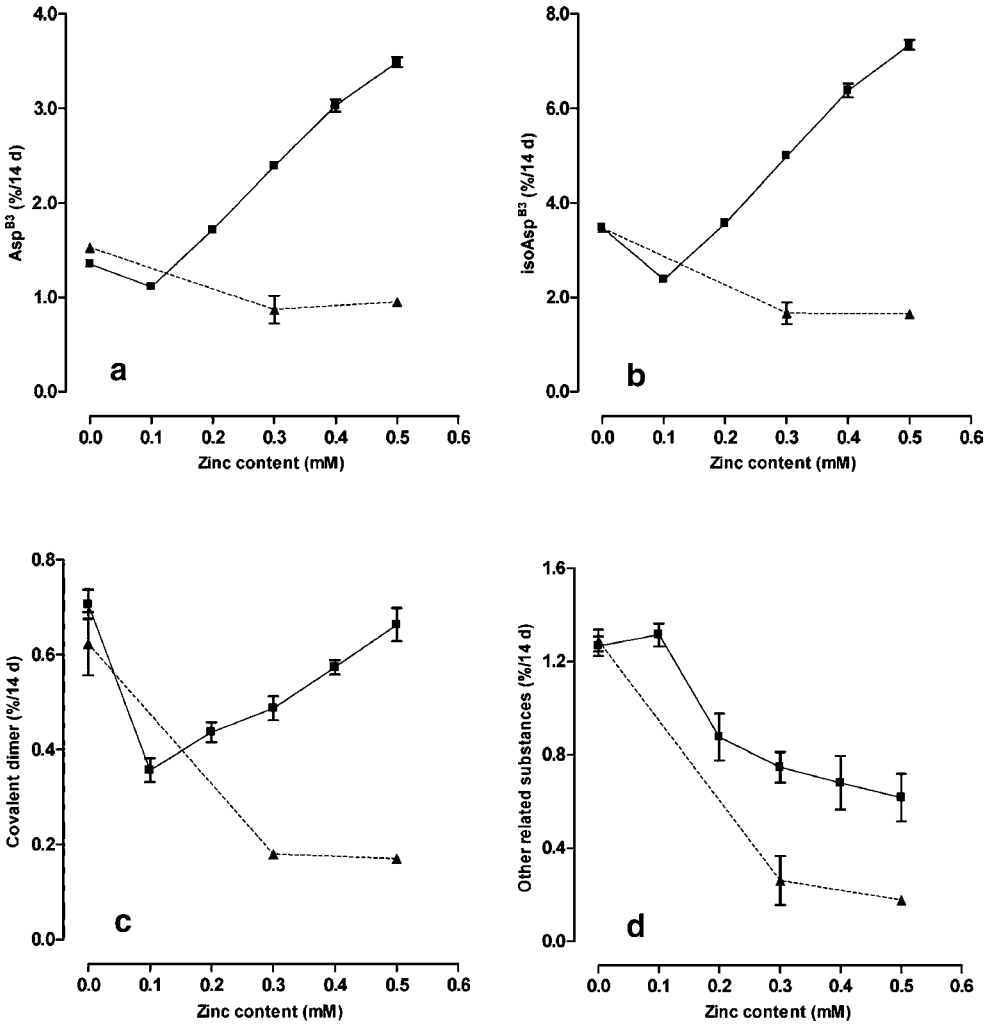

Fig. 1. Effect of zinc ions on the B3 deamidation of human insulin. Formation of Asp^{B3} (a), isoAsp^{B3} (b), covalent dimer (c), and other insulin related substances (d) after 14 days at 37°C. The samples contained 0.6 mM human insulin in 7 mM phosphate buffer at pH 7.4 with 20 mM phenol (▲) or without phenol (■). Error bars are included to indicate standard deviation (n = 3).

bance was used to calculate the molar ellipticity (θ) on a per residue basis.

RESULTS

Effect of Zinc on Insulin Chemical Stability

The conversion rate of Asn^{B3} to Asp^{B3} and isoAsp^{B3} at different concentrations of zinc was followed by RP-HPLC. The amount of deamidation products formed after 14 days at 37°C in the absence and presence of 20 mM phenol at 0 to 5 Zn^{2+}/hexamer is shown in Fig. 1. In the absence of phenol, addition of 1 Zn^{2+}/hexamer slightly stabilizes against deamidation. However, at increasing zinc concentrations, the deamidation rate increased linearly up to 5 Zn^{2+}/hexamer. In contrast, in the presence of phenol, the deamidation rate remained constant when the zinc concentration is increased from 3 to 5 Zn^{2+}/hexamer.

No polymer products larger than dimer were observed in these samples or in any of the other samples in this study. The formation of covalent dimer followed the same trend as the deamidation as a function of zinc and phenol content. On the other hand, the formation rate of other insulin related substances decreased when the zinc concentration was increased from 0 to 2 Zn^{2+}/hexamer. A further increase in

Table I. Effect of Zinc Ions on the Formation of Fragments Smaller than Monomeric Insulin

	Formation Rate of Fragments (%/14 days)	
Zn^{2+}/hexamer	Without phenol	20 mM phenol
0.0	0.36 ± 0.01	0.35 ± 0.02
1.0	0.21 ± 0.01	–
2.0	0.16 ± 0.01	–
3.0	0.12 ± 0.02	0.0[a]
4.0	0.10 ± 0.01	–
5.0	0.05 ± 0.01	0.0[a]

The amount of fragments formed after 14 days at 37°C in the absence or presence of 20 mM phenol and 0–5 Zn^{2+} /hexamer was determined by SEC. The results are shown with standard deviation (n = 3).
[a] No fragments were observed in the SEC-analyses.

the zinc concentration did not affect the formation of other insulin related substances. The same pattern was observed in the presence of phenol, where the degradation was even slower (Fig. 1).

A small amount of substances smaller than insulin monomer was observed by SEC (Table I). Analysis by MALDI-TOF MS revealed species corresponding to the B-chain of insulin (the A-chain is usually not detected when the B-chain is present in the same sample). The monoisotopic molecular weight of the detected fragment was 3,425.7 Da (data not shown), which is 2 Da less than the theoretical weight of the B-chain. This means, that the B-chain is most likely oxidized and contains an intrachain disulfide bridge. The formation rate of fragments in the zinc-free samples was about 0.36%/14 days. With increasing zinc concentrations, the degree of fragmentation decreased and in the presence of 5 Zn^{2+}/hexamer the rate was only 0.05%/14 days. In the presence of 3 or 5 Zn^{2+}/hexamer and 20 mM phenol no fragments were detected.

Effect of Phenolic and Anionic Ligands on Insulin Chemical and Thermal Stability

The effect of the different ligands on the biphasic thermal denaturation profile of human insulin was examined by DSC. Typical DSC thermograms which illustrate the effect of adding phenol and a combination of phenol and chloride to human insulin with 3 Zn^{2+}/hexamer are shown in Fig. 2. There are two distinct peaks in the DSC thermograms, and these have maximal heat capacities at $T_{max,1}$ and $T_{max,2}$ for the low and high temperature peaks, respectively.

Results of $T_{max,1}$ and $T_{max,2}$ from all DSC experiments with the different ligands are shown in Fig. 3. $T_{max,1}$ is slightly

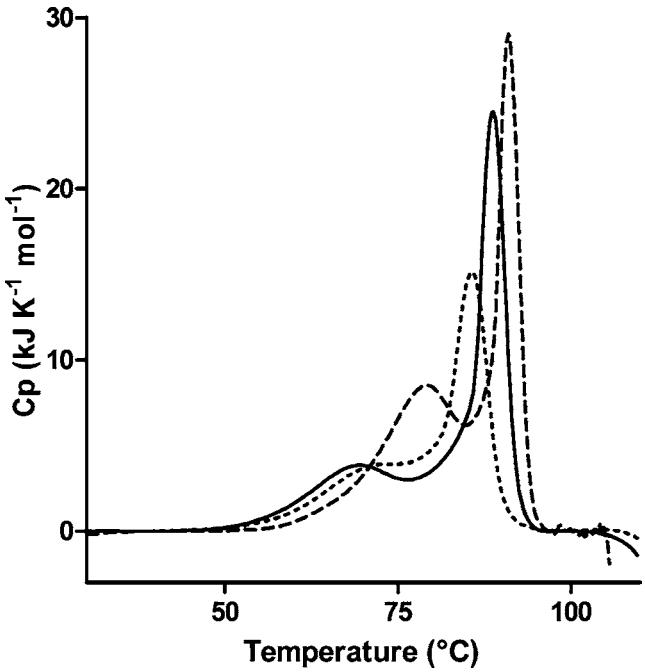

Fig. 2. Effect of ligands on the biphasic thermal denaturation of human insulin. DSC thermograms of 0.6 mM human insulin with 3 Zn^{2+}/hexamer in 7 mM phosphate buffer at pH 7.4. *Dotted line*, reference without ligands; *full line*, 20 mM phenol; *dashed line*, 20 mM phenol + 50 mM NaCl.

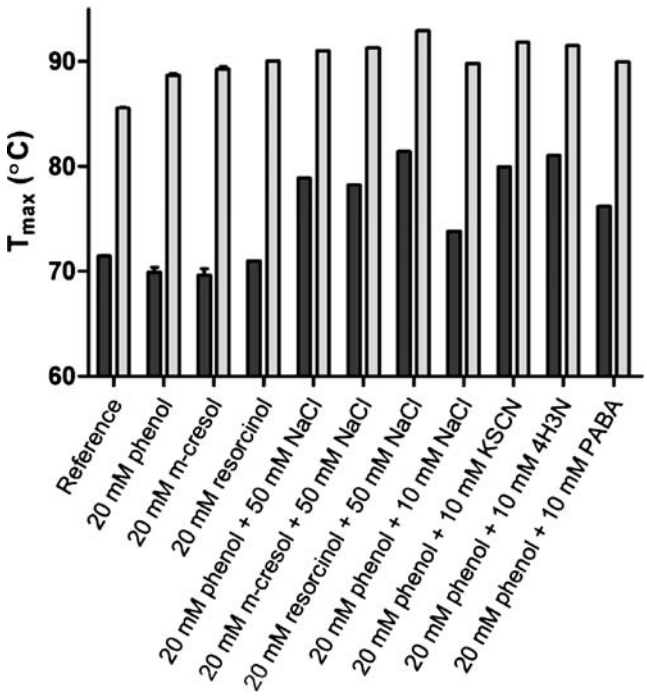

Fig. 3. Effect of different ligands on the thermal denaturation of human insulin. DSC experiments were performed with 0.6 mM human insulin with 3 Zn^{2+}/hexamer in 7 mM phosphate buffer at pH 7.4. The added ligands are indicated in the figure, which shows $T_{max,1}$ (*dark bars*) and $T_{max,2}$ (*light bars*) of the biphasic denaturation.

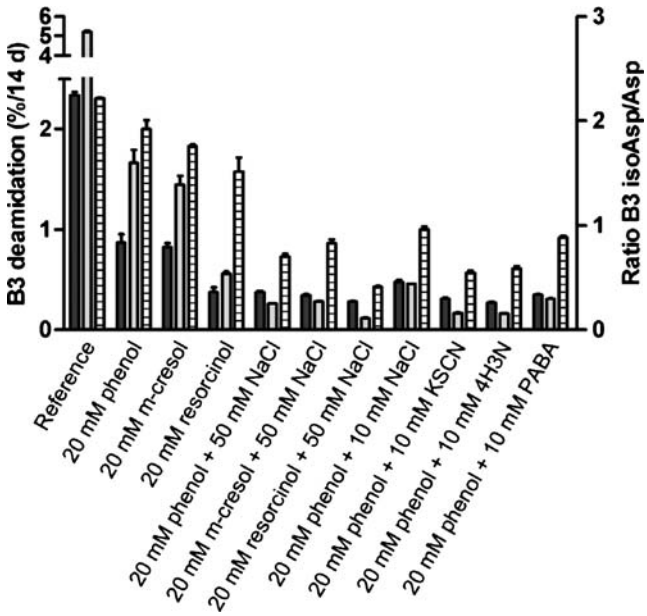

Fig. 4. Effect of ligands on the B3 deamidation of human insulin. Samples containing 0.6 mM human insulin with 3 Zn^{2+}/hexamer in 7 mM phosphate buffer at pH 7.4 were stored at 37°C for 14 days prior to analysis. The added ligands are indicated in the figure, which shows the formation of B3 deamidation products (*left y-axis*) and the ratio isoAsp/Asp (*right y-axis*). *Dark bars*, Asp^{B3}; *light bars*, $isoAsp^{B3}$; *striped bars*, isoAsp/Asp ratio. *Error bars* indicate the standard deviation (n = 3).

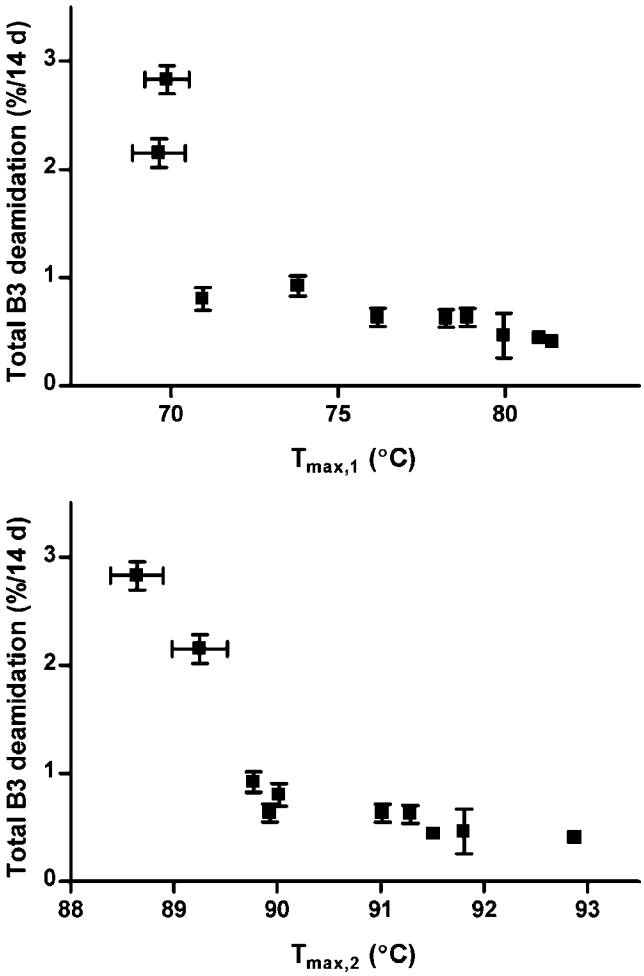

Fig. 5. Correlation between the total amounts of B3 deamidation products formed and $T_{max,1}$ (*top graph*) and $T_{max,2}$ (*bottom graph*) from DSC experiments.

reduced by the phenolic ligands, but $T_{max,2}$ is significantly increased in the order resorcinol >*m*-cresol ≥ phenol, when compared to the reference without ligands. When 50 mM chloride is present, the increase in $T_{max,2}$ is larger for all three phenolic ligands. The presence of 50 mM chloride with the 3 phenolic ligands also leads to remarkable increases in $T_{max,1}$ in the order resorcinol > phenol ≥ *m*-cresol. Four anionic ligands were tested in the presence of 20 mM phenol. They increased $T_{max,1}$ in the order 4H3N > SCN^- > PABA > Cl^-, and T_{max2} in the order SCN^- ≥ 4H3N > PABA > Cl^-.

Addition of the various ligands had a pronounced effect on the conversion rate of Asn^{B3} to Asp^{B3} and $isoAsp^{B3}$ (see Fig. 4). The decrease in deamidation rates followed the same order for the ligands as observed for the stabilization in the DSC studies. The correlation between $T_{max,2}$ and deamidation rate was slightly better than the correlation between $T_{max,1}$ and deamidation rate (Fig. 5).

The formation rate of covalent dimer did not follow the same order as the formation of deamidation products. All ligands decreased the dimerization rate significantly compared to the reference (see Fig. 6). However, phenol gave the best stabilization against dimerization (reduced from 0.53 to 0.18%/14 days), whereas the rate was only reduced to 0.29 and 0.23%/14 days with *m*-cresol and resorcinol, respectively. In the presence of 50 mM NaCl, the formation of covalent dimer was higher with resorcinol than with phenol and *m*-cresol. The addition of anionic ligands in the presence of 20 mM phenol further reduced the dimerization in the order PABA > Cl^- = 4H3N ≥ SCN^-. The formation of other related substances was reduced by all ligands except with resorcinol (see Fig. 6). The combination of resorcinol and NaCl caused a dramatic increase in formation of other related substances. After 14 days at 37°C, 9.0% was formed compared to only 0.54% for the reference. There was no significant difference between phenol and *m*-cresol, or between the four anionic ligands.

Effect of Thiocyanate on Insulin in the Absence of Phenol

The changes induced by thiocyanate in the NUV-CD signal of zinc-insulin are shown in Fig. 7. The intensity of the negative CD-signal at 276 nm was decreased while the intensity of the negative CD-signal at around 255 nm was increased. The changes in ellipticity around 255 nm are caused by changes in the disulfide dihedral angles connected with the T to R transition (23). Thiocyanate drives the equilibrium of the insulin hexamer towards predominantly T_3R_3 (24–26). The signal at 276 nm originates from the tyrosine side-chains in interfaces in the dimer and in the hexamer, and apparently this signal is also influenced by the binding of thiocyanate (27,28). A series with increasing thiocyanate concentrations showed a saturation of the changes in the 255 nm signal at around 50 mM thiocyanate, while the changes in the signal at 276 nm were more subtle (see Fig. 7b). NUV-CD spectra were collected during temperature scans with samples with and without thiocyanate

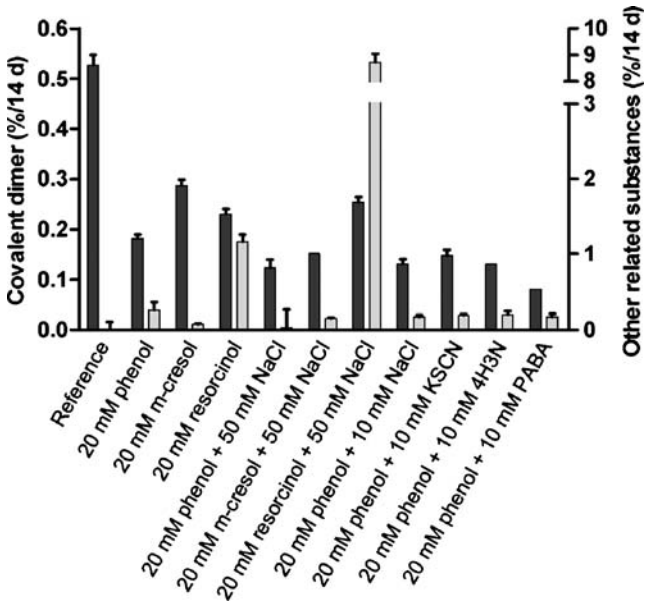

Fig. 6. Effect of ligands on the formation of covalent dimer and other related substances. Samples containing 0.6 mM human insulin with 3 Zn^{2+}/hexamer in 7 mM phosphate buffer at pH 7.4 were stored at 37°C for 14 days prior to analysis. The added ligands are indicated in the figure, which shows the formation of covalent dimer (*dark bars and left y-axis*) and the formation of other related substances (*light bars and right y-axis*). *Error bars* indicate the standard deviation (n = 3).

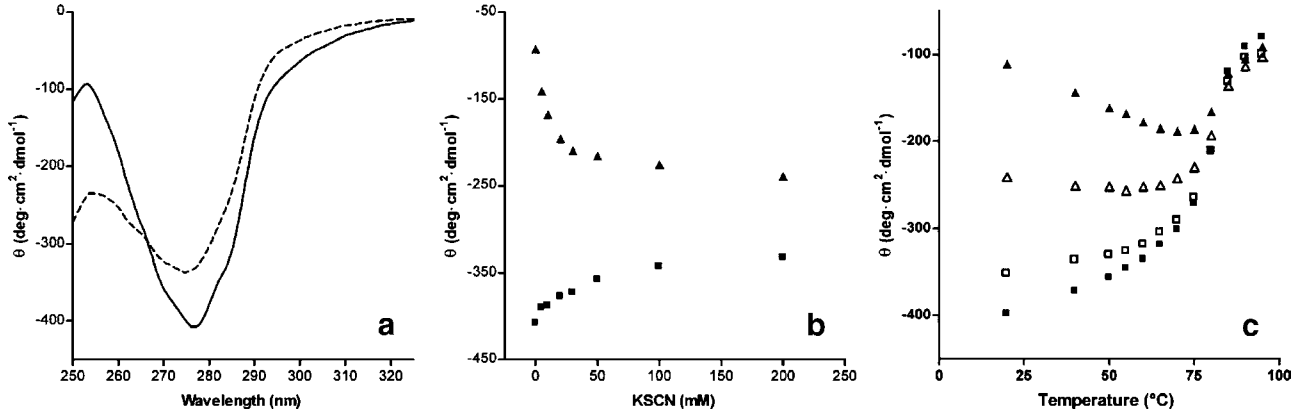

Fig. 7. Effect of thiocyanate and heating on the NUV-CD spectrum of human insulin. Samples contained 0.6 mM human insulin with 5 Zn^{2+}/hexamer in 7 mM phosphate at pH 7.4. (a) Spectra collected in the absence (*full line*) and presence of 200 mM KSCN (*dashed line*). (b) The effect of increasing thiocyanate concentrations on the NUV-CD signal at 255 nm (▲) and 276 nm (■). (c) Effect of heating on the NUV-CD signal at 255 nm (*triangles*) and 276 nm (*squares*) in the absence (*filled symbols*) and presence of 100 mM thiocyanate (*open symbols*).

(see Fig. 7c) and the CD-signals at 255 and 276 nm were followed. At both wavelengths a transition is observed at approximately 85°C. The NUV-CD signals of the samples with and without thiocyanate approached each other in the course of the heating. Immediately before the transition, the 276 nm signals are identical and the 255 nm signals are almost the same.

The effect of thiocyanate on the thermal denaturation of insulin was studied by DSC with 3 and 5 Zn^{2+}/hexamer. With 3 Zn^{2+}/hexamer and 50 mM KSCN the thermal denaturation was biphasic (similar to Fig. 2). We observed a slight decrease in the enthalpy of Peak 1 and a slight increase in the enthalpy of peak 2, but $T_{max,2}$ remained unchanged compared to a thiocyanate-free reference (data not shown).

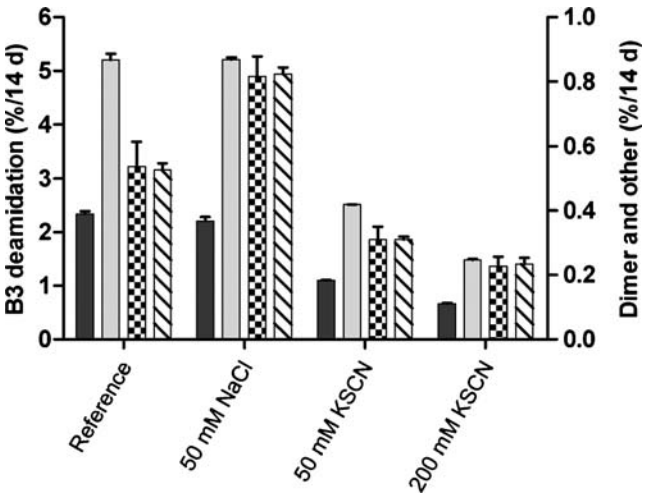

Fig. 8. Effect of NaCl and KSCN on the chemical stability of human insulin. Samples containing 0.6 mM human insulin with 3 Zn^{2+}/hexamer in 7 mM phosphate buffer at pH 7.4 were stored at 37°C for 14 days prior to analysis. The added ligands are indicated in the figure, which shows the formation of B3 deamidation products (*left y-axis*) and covalent dimer and other related substances (*right y-axis*). *Dark bars*, Asp^{B3}; *light bars*, $isoAsp^{B3}$; *checkered bars*, total other related; *striped bars*, covalent dimer. *Error bars* indicate the standard deviation (n = 3).

With 5 Zn^{2+}/hexamer and up 200 mM KSCN the thermal denaturation only consisted of the hexamer unfolding peak. We did not observe any change in either the enthalpy or T_{max} when compared to a thiocyanate-free reference (data not shown). The presence of 400 mM KSCN caused some distortion of the thermograms and slightly lowered the T_{max}. In contrast, it was found that thiocyanate increased the stability against chemical degradation significantly (see Fig. 8). Both the formation rates of Asp^{B3} and $isoAsp^{B3}$ were more than halved in the presence of 50 mM thiocyanate and even further reduced with 200 mM thiocyanate. The Asp^{B3}/$isoAsp^{B3}$ ratio was not influenced by the presence of thiocyanate. A ligand-free sample was added 50 mM chloride to control if the ionic strength obtained by 50 mM KSCN influenced the deamidation rate. The degradation rate of the sample with 50 mM NaCl was similar to the ligand-free reference and therefore, it was concluded that the effect of KSCN on deamidation was not related to the increased ionic strength. The formation rate of covalent dimer and other insulin related degradation substances were reduced in the presence of thiocyanate by relative amounts similar to the deamidation rate. In contrast, the formation rate was increased in the presence of 50 mM chloride. The formation rates of covalent dimer and total other insulin related substances were similar, indicating that all other insulin related substances in these samples were covalent dimer.

DISCUSSION

Effect of Zinc on Insulin Chemical Stability

Surprisingly, a significant increase in B3 deamidation was observed with increased zinc concentrations in the absence of phenolic preservatives. It has previously been stated that zinc does not have any influence on the chemical stability of insulin. Thus, Brange *et al.* (29) did not find any significant difference in the chemical stability between 2 and 4 Zn^{2+}/hexamer in formulations with porcine or human insulin. However, their formulations contained 0.2% (or 21.3 mM) phenol. In accordance with those findings, our

results showed no effect of increasing the zinc content from 3 to 5 Zn^{2+}/hexamer in the presence of 20 mM phenol. Thus, apparently zinc accelerates B3 deamidation in the insulin T_6-hexamer but not in the R_6-hexamer (i.e., in the presence of phenol).

In the absence of Zn^{2+}, the isoAsp/Asp ratio is 2.6:1, which is close to the ratio of 3:1, often found in various other systems (30). The ratio was decreased to around 2.1:1 with 1 Zn^{2+}/hexamer. Further increases in the Zn^{2+} concentration significantly increased the deamidation but the isoAsp/Asp ratio remained unchanged at 2.1. Thus, the mechanism of the increased deamidation caused by Zn^{2+} is not specific to either of the two B3 deamidation products.

When Zn^{2+} is added, the first sites to be occupied are the two central sites, which organize the hexamer (31–33). The affinity of Zn^{2+} to the hexamer-coordinating site is around 10^6 M^{-1} at pH 7–8 and 20–30°C (31,32,34). Therefore, in the present study, when the total Zn^{2+}/insulin ratio is 1 Zn^{2+}/hexamer (0.1 mM Zn^{2+} and 0.6 mM insulin), we can assume that most Zn^{2+} is bound in insulin hexamers. Thus, almost half the insulin population will be hexameric and the rest dimeric. Since 1 Zn^{2+}/hexamer decreased the deamidation rate, we conclude that insulin in the hexamer state is stabilized against B3 deamidation and that when bound at the hexamer-coordinating sites, zinc ions do not increase the B3 deamidation. The increased deamidation at higher zinc ion concentrations must therefore be due to either zinc binding in other sites or free zinc ions. At increasing concentrations, zinc binds in various sites, the most fully occupied being in the hexamer centre between the 6 Glu^{B13} residues. Additional binding primarily involves interactions with Glu and His residues on the surface of the hexamer (33). In the T_6 hexamer, the His^{B5} and Glu^{B21} side chains are both within a 5 Å distance of Asn^{B3} and the Asn^{B3} side-chain carbonyl is accessible to the surroundings. In the R_6 hexamer, however, the His^{B5} and Glu^{B21} side chains are both further away from Asn^{B3} and the access is sterically hindered. We propose that Zn^{2+} increases the susceptibility of Asn^{B3}-deamidation in the T_6 hexamer by coordinating to the Asn^{B3} side-chain carbonyl and either His^{B5} or Glu^{B21} side-chain and water molecules or possibly just by coordinating to the Asn^{B3} side-chain carbonyl and water molecules. This will render the Asn^{B3} side-chain carbonyl more susceptible towards nucleophilic attack from the Asn^{B3} backbone nitrogen leading to deamidation through the cyclic imide intermediate. In the R_6 hexamer, the structure around Asn^{B3} prevents interaction between Zn^{2+} and the Asn^{B3} side-chain carbonyl.

The influence of zinc and phenol on the relative changes in the formation rate of covalent insulin dimer was identical to the changes in B3 deamidation rate. It is assumed that covalent insulin dimer formed in neutral insulin preparations consist mainly (2/3) of products of reactions between the B-chain N-terminal and with side-chain amido groups in the A-chain (most likely Gln^{A15}, Asn^{A18}, and Asn^{A21}) of another insulin molecule. The remaining (1/3) covalent insulin dimer are the products of similar reactions between the A-chain N-terminal and similar amido groups in another A-chain (35,36). Our results with phenol show that the covalent insulin dimer formation is reduced to approximately 1/3 in the presence of 3 or 5 Zn^{2+}/hexamer compared to the zinc-free samples. This stabilization is presumably due to the decreased flexibility in the B1–B8 segment of the R-state hexamer, thus decreasing the possibilities of B1 to react with other groups. Similar results have been obtained with porcine insulin in the presence of methylparaben (37) and with human insulin and the analogue $Lys^{B28}Pro^{B29}$-insulin with phenol and *m*-cresol (38). In the absence of phenolic ligands, we found that the formation rate of covalent insulin dimer increased with increasing zinc concentrations. We hypothesize that zinc, analogous to the effect on deamidation, increases the susceptibility towards nucleophilic attack in the dimerization reaction.

In contrast to the detrimental effect of zinc with regard to dimerization and B3 deamidation, it was shown that increasing zinc concentrations decreased the rate of formation of insulin fragments. Apparently, the disulfide bonds between the A- and B-chain were broken resulting in insulin fragments consisting of A- and B-chain. The molecular weight of the B-chain indicated that the free cystein residues were oxidized and formed an intrachain disulfide bridge. Disulfide-scrambling is a common phenomenon in proteins, but it is normally not observed in pharmaceutical preparations of insulin (35). Fragments smaller than the insulin monomer have previously been detected in insulin formulations containing phenol at pH values above 8 (37) and in samples of zinc-free insulin analogues (18). The fragments were suggested to consist of insulin A- and B-chains, but no data concerning the identity of the fragments were shown (18,37). Breakage and formation of new disulfide has been elucidated in lyophilized insulin and it was shown to occur primarily in the presence of hydroxide ions (39). The increased stability against fragmentation with increased zinc concentration indicates that disulfide-scrambling is less likely to occur in the insulin hexamer. When the zinc-hexamer was further stabilized with phenol, no fragments were observed at all. Insulin preparations generally contain zinc and one or more phenolic preservatives and with pH adjusted around 7.4. Therefore, according to the present results, due to the stability of ligand-bound insulin zinc-hexamer, disulfide-scrambling is not likely to occur and consequently fragments are not normally observed in pharmaceutical preparations.

Effects of Phenolic and Anionic Ligands on Insulin Stability

Thermal Stability

We have previously shown that more than 4 Zn^{2+}/hexamer are required to observe a monophasic thermal denaturation of hexameric insulin (5). However, soluble insulin formulations typically contain only 2–3 Zn^{2+}/hexamer in order to reduce the risk of insulin crystallization during storage. We have previously used DSC to study the influence of phenolic and anionic ligands on the hexameric zinc-insulin system with 5 Zn^{2+}/hexamer (6). The present DSC studies were all performed with 3 Zn^{2+}/hexamer to evaluate the applicability of DSC studies of insulin with pharmaceutically relevant zinc concentrations. This lower zinc concentration gives rise to a biphasic thermal denaturation profile with two distinct peaks and it was the purpose of the present study to evaluate the effects of ligands on the two peaks.

Peak 2 represents the concurrent dissociation and unfolding of hexameric insulin. The stabilizing effect of the

ligands caused increases in $T_{max,2}$, in an order which corresponded exactly to the order of affinity previously found in the system with 5 Zn^{2+}/hexamer (6). The effects of the ligands on $T_{max,1}$ were more complicated. Phenol and *m*-cresol caused very slight decreases in $T_{max,1}$ while it was unchanged by resorcinol. This can be explained by the destabilizing effect on proteins often observed for phenolic preservatives, as previously shown for zinc-free insulin (6). On the other hand, the anionic ligands (in the presence of phenol) all gave rise to significant increases in $T_{max,1}$. The effect was only observed in the presence of phenolic ligand and the increases in $T_{max,1}$ correlated to the affinity of the ligands to the insulin zinc-hexamer (except that 4H3N gave a slightly higher increase than KSCN). This is a very strong indication, that the increases in $T_{max,1}$ are caused by increased stability of the zinc-coordination induced by the anionic ligands, which corresponds to increasing the affinity of zinc. According to the mechanism of ligand-induced biphasic denaturation, stabilization of the ligand-protein complex (in this case Zn^{2+}-insulin) will increase both $T_{max,1}$ and $T_{max,2}$ because the coupled equilibriums of ligand binding and protein unfolding are shifted towards higher temperature (40).

Chemical Stability

The reduction in AsnB3 deamidation caused by the different ligands was found to follow the same order as the increases in T_{max}, caused by the same ligands. The reduced deamidation is caused by the stabilization of the R conformation state by the ligands. In the R state, the conformational flexibility around Asn^{B3} is considerably reduced, making the deamidation less likely (36). Furthermore, occupancy of the binding site of the anionic ligands causes a steric hindrance around the Asn^{B3} sidechains which might also influence the deamidation process. The ratio between isoAsp and Asp formation is dependent on the structure surrounding the Asn residue. When the structural flexibility around the Asn residue is high, the isoAsp/Asp ratio is typically around 3:1 (30). In the B3 deamidation of the ligand free reference samples with 3 Zn^{2+}/hexamer we obtained an isoAsp/Asp ratio around 2:1. With increased ligand stabilization we found significant reductions in the isoAsp/Asp ratio. The decrease in the ratio followed the order of stabilization found with DSC with both the phenolic ligands (with the exception of 20 mM *m*-cresol + 50 mM NaCl having a slightly higher ratio than 20 mM phenol + 50 mM NaCl) and the anionic ligands (in the presence of phenol). In the most stable samples less $isoAsp^{B3}$ than Asp^{B3} was formed. According to Brange (36), the most probable explanation of the reduced formation in the R-state of $isoAsp^{B3}$ relative to Asp^{B3}, is hydrogen bonding to one of the two oxygen in the cyclic imide intermediate. This hydrogen bonding may occur when α-helix is induced in the residues 1–8 in the B-chain, as is the case in the R-state of the insulin hexamer. If hydrogen bonds are formed to the former Asn^{B3} side-chain carbonyl in the imide intermediate, then a larger amount of Asp^{B3} relative to $isoAsp^{B3}$ will be formed (36).

Whereas the B3 deamidation rate correlated to the affinity of the ligands as found by DSC, the formation rates of covalent dimer and other related substances did not correlate to the affinity order. Stabilization was observed in all cases, except in the presence of resorcinol. The different ligands and combinations of ligands caused seemingly random changes in insulin degradation. Most notably, the combination of resorcinol and NaCl caused an almost 20-fold increase in the formation rate of other insulin related substances. The exact mechanism is unknown, but the increased degradation in the presence of resorcinol may be caused by reactive oxidation products of resorcinol. Phenolic compounds are readily oxidized to quinones in the presence of molecular oxygen, and due to the extra hydroxyl group, resorcinol is more easily oxidized to quinone than phenol and *m*-cresol are. Quinones are highly reactive compounds that react primarily with amino groups of proteins. Quinones and their reaction products are coloured substances (41–43). Coloration was observed in the samples with resorcinol after 14 days at 37°C. It is therefore not unlikely that the degradation was increased due to quinone oxidation products of resorcinol and apparently this was accelerated in the presence of NaCl.

Thiocyanate Interactions with Insulin

The thiocyanate concentration dependence of the changes in the NUV-CD spectra (Fig. 7b) correlate well with previous work, which states that 30–50 mM thiocyanate induces the conversion T_6 to T_3R_3 in human insulin. Saturation of the conversion occurs at KSCN concentrations around 200–400 mM (19,23–26,44,45). Our DSC studies showed that thiocyanate did not improve the thermal stability of the zinc-hexamer, indicating that thiocyanate does not bind to the hexamer at the T_{max} temperature and that the unfolding of T_6 rather than T_3R_3 is observed. On the other hand, the results of the chemical stability studies showed a significant reduction in the B3 deamidation in the presence of thiocyanate, indicating stabilization of R-state in the insulin hexamer. These conflicting results are explained by the differences in temperature, and hence differences in the binding affinity of thiocyanate, between the NUV-CD binding study (20°C), the chemical stability study (37°C), and the DSC experiments (with denaturation at 75–95°C). The temperature effect is clear in Fig. 7c, which shows that the effect of thiocyanate diminishes as the temperature is increased.

Even though the B3 deamidation was significantly reduced, the Asp^{B3}/$isoAsp^{B3}$ ratio remained unchanged in the presence of thiocyanate. This is in contrast to the results obtained with phenol bound R_6-hexamer, where the $isoAsp^{B3}$/Asp^{B3} ratio was drastically reduced. It has been found in thiocyanate-bound T_3R_3 crystals (46,47) and in solution (45), that B1–B3 do not adapt α-helical structure in the R-units. This may explain why thiocyanate stabilizes against B3-deamidation but does not reduce the Asp^{B3}/$isoAsp^{B3}$ ratio as observed with the R_6-hexamer in the presence of phenolic ligands.

CONCLUSION

We conclude that the biphasic thermal denaturation profile of insulin that is observed with pharmaceutically relevant zinc concentrations may be used to predict insulin

stability. The values of $T_{max,2}$ correlated excellently to the conformational state of the hexamer and to the ligand affinities. The values of $T_{max,1}$ partly correlated to the hexamer conformation and ligand affinity because it was under influence of dimer destabilization caused by the phenolic ligands. The correlation between the DSC results and the major insulin degradation products (B3 deamidation products) was excellent, but the formation of covalent dimer and other insulin related substances did not show exactly the same relationship. The specific binding of both phenolic and anionic ligands stabilizes the R_6 structure and decreases the flexibility around the Asn^{B3} by inducing α-helix in residues B1–B8. Even though this stabilization increased the overall hexamer stability (as determined by DSC) it did not necessarily predict the rate of formation of insulin dimer and other related substances. Our findings of the influence of zinc in the absence of phenolic preservatives were surprising. These findings are important to take into account if formulations without phenolic preservatives are being developed. The results with thiocyanate show that even though DSC is an excellent tool for protein stability screening, it does not necessarily predict the stability at lower temperatures. Care should be exercised in the interpretation of effects on degradation processes which are independent of the structural stability. In principle, these are not expected to correlate to the stability as determined by DSC.

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REFERENCES

1. J. Brange. *Stability of Insulin-Studies on the Physical and Chemical Stability of Insulin in Pharmaceutical Formulation*, Kluwer, Boston, 1994.
2. Q. X. Hua and M. A. Weiss. Mechanism of insulin fibrillation: the structure of insulin under amyloidogenic conditions resembles a protein-folding intermediate. *J. Biol. Chem.* **279**:21449–21460 (2004).
3. L. Nielsen, R. Khurana, A. Coats, S. Frokjaer, J. Brange, S. Vyas, V. N. Uversky, and A. L. Fink. Effect of environmental factors on the kinetics of insulin fibril formation: Elucidation of the molecular mechanism. *Biochemistry* **40**:6036–6046 (2001).
4. J. Brange and L. Langkjaer. Insulin structure and stability. *Pharm. Biotechnol.* **5**:315–350 (1993).
5. K. Huus, S. Havelund, H. B. Olsen, M. van de Weert, and S. Frokjaer. Thermal dissociation and unfolding of insulin. *Biochemistry* **44**:11171–11177 (2005).
6. K. Huus, S. Havelund, H. B. Olsen, B. W. Sigurskjold, M. van de Weert, and S. Frokjaer. Ligand binding and thermostability of different allosteric states of the insulin zinc-hexamer. *Biochemistry* **45**:4014–4024 (2006).
7. R. L. Remmele and W. R. Gombotz. Differential scanning calorimetry: A practical tool for elucidating stability of liquid biopharmaceuticals. *Biopharm.* **13**:36–46 (2000).
8. R. L. Remmele Jr., N. S. Nightlinger, S. Srinivasan, and W. R. Gombotz. Interleukin-1 receptor (IL-1R) liquid formulation development using differential scanning calorimetry. *Pharm. Res.* **15**:200–208 (1997).
9. F. K. Bedu-Addo, C. Johnson, S. Jeyarajah, I. Henderson, and S. J. Advant. Use of biophysical characterization in preformulation development of a heavy-chain fragment of botulinum serotype B: evaluation of suitable purification process conditions. *Pharm. Res.* **21**:1353–1361 (2004).
10. Y. F. Maa and C. C. Hsu. Aggregation of recombinant human growth hormone induced by phenolic compounds. *Int. J. Pharm.* **140**:155–168 (1996).
11. Y. Zhang, S. Roy, L. S. Jones, S. Krishnan, B. A. Kerwin, B. S. Chang, M. C. Manning, T. W. Randolph, and J. F. Carpenter. Mechanism for benzyl alcohol-induced aggregation of recombinant human interleukin-1 receptor antagonist in aqueous solution. *J. Pharm. Sci.* **93**:3076–3089 (2004).
12. S. Gupta and E. Kaisheva. Development of a multidose formulation for a humanized monoclonal antibody using experimental design techniques. *AAPS PharmSci.* **5**:E8 2003 (2003).
13. W. Wang. Instability, stabilization, and formulation of liquid protein pharmaceuticals. *Int. J. Pharm.* **185**:129–188 (1999).
14. M. Xie. Secondary structure and protein deamidation. *J. Pharm. Sci.* **88**:8–13 (1999).
15. A. A. Kossiakoff. Tertiary structure is a principal determinant to protein deamidation. *Science* **240**:191–194 (1988).
16. N. E. Robinson and A. B. Robinson. Prediction of protein deamidation rates from primary and three-dimensional structure. *Proc. Natl. Acad. Sci. USA* **98**:4367–4372 (2001).
17. S. J. Wearne and T. E. Creighton. Effect of protein conformation on rate of deamidation: ribonuclease A. *Proteins* **5**:8–12 (1989).
18. D. N. Brems, P. L. Brown, C. Bryant, R. E. Chance, L. K. Green, H. B. Long, A. A. Miller, R. Millican, J. E. Shields, and B. H. Frank. Improved insulin stability through amino acid substitution. *Protein Eng.* **5**:519–525 (1992).
19. S. Rahuel-Clermont, C. A. French, C. I. Chou, N. C. Kaarsholm, and M. F. Dunn. Mechanisms of stabilization of the insulin hexamer through allosteric ligand interactions. *Biochemistry* **36**:5837–5845 (1997).
20. J. Brange, U. Ribel, J. F. Hansen, G. Dodson, M. T. Hansen, S. Havelund, S. G. Melberg, F. Norris, K. Norris, L. Snel, A. R. Sørensen, and H. O. Voigt. Monomeric insulins obtained by protein engineering and their medical implications. *Nature* **333**:679–682 (1988).
21. M. U. Jars, A. Hvass, and D. Waaben. Insulin aspart (Asp(B28) human insulin) derivatives formed in pharmaceutical solutions. *Pharm. Res.* **19**:621–628 (2002).
22. V. V. Plotnikov, J. M. Brandts, L. N. Lin, and J. F. Brandts. A new ultrasensitive scanning calorimeter. *Anal. Biochem.* **250**: 237–244 (1997).
23. J. Shneine, M. Voswinkel, M. Federwisch, and A. Wollmer. Enhancing the T → R transition of insulin by helix-promoting sequence modifications at the N-terminal B-chain. *Biol. Chem.* **381**:127–133 (2000).
24. M. L. Brader, N. C. Kaarsholm, R. W. Lee, and M. F. Dunn. Characterization of the R-state insulin hexamer and its derivatives. The hexamer is stabilized by heterotropic ligand binding interactions. *Biochemistry* **30**:6636–6645 (1991).
25. W. E. Choi, M. L. Brader, V. Aguilar, N. C. Kaarsholm, and M. F. Dunn. The allosteric transition of the insulin hexamer is modulated by homotropic and heterotropic interactions. *Biochemistry* **32**:11638–11645 (1993).
26. N. C. Kaarsholm, H. C. Ko, and M. F. Dunn. Comparison of solution structural flexibility and zinc binding domains for insulin, proinsulin, and miniproinsulin. *Biochemistry* **28** 4427–4435 (1989).
27. E. H. Strickland and D. Mercola. Near-ultraviolet tyrosyl circular dichroism of pig insulin monomers, dimers, and hexamers. Dipole-dipole coupling calculations in the monopole approximation. *Biochemistry* **15**:3875–3884 (1976).
28. A. Ahmad, I. S. Millett, S. Doniach, V. N. Uversky, and A. L. Fink. Partially folded intermediates in insulin fibrillation. *Biochemistry* **42**:11404–11416 (2003).
29. J. Brange, S. Havelund, E. Hommel, E. Sørensen, and C. Kühl. Neutral insulin solutions physically stabilized by addition of Zn^{2+}. *Diabet. Med.* **3**:532–536 (1986).
30. T. V. Brennan and S. Clarke. Deamidation and isoaspartate formation in model synthetic peptides: The effects of sequence and solution environment. In D. W. Aswad (ed.), *Deamidation and Isoaspartate Formation in Peptides and Proteins*, CRC Press, Boca Raton, 1995, pp. 65–90.

31. P. T. Grant, T. L. Coombs, and B. H. Frank. Differences in the nature of the interaction of insulin and proinsulin with zinc. *Biochem. J.* **126**:433–440 (1972).
32. J. M. Summerell, A. Osmand, and G. H. Smith. An equilibrium-dialysis study of binding of zinc to insulin. *Biochem. J.* **95**:31 (1965).
33. S. O. Emdin, G. G. Dodson, J. M. Cutfield, and S. M. Cutfield. Role of zinc in insulin biosynthesis. Some possible zinc-insulin interactions in the pancreatic B-cell. *Diabetologia* **19**:174–182 (1980).
34. J. Goldman and F. H. Carpenter. Zinc binding, circular dichroism, and equilibrium sedimentation studies on insulin (bovine) and several of its derivatives. *Biochemistry* **13**: 4566–4574 (1974).
35. J. Brange, O. Hallund, and E. Sørensen. Chemical stability of insulin. 5. Isolation, characterization and identification of insulin transformation products. *Acta Pharm. Nord.* **4**:223–232 (1992).
36. J. Brange. Chemical stability of insulin. 4. Mechanisms and kinetics of chemical transformations in pharmaceutical formulation. *Acta Pharm. Nord.* **4**:209–222 (1992).
37. J. Brange and L. Langkjaer. Chemical stability of insulin. 3. Influence of excipients, formulation, and pH. *Acta Pharm. Nord.* **4**:149–158 (1992).
38. D. L. Bakaysa, J. Radziuk, H. A. Havel, M. L. Brader, S. Li, S. W. Dodd, J. M. Beals, A. H. Pekar, and D. N. Brems. Physicochemical basis for the rapid time-action of LysB28-ProB29-insulin: dissociation of a protein-ligand complex. *Protein Sci.* **5**:2521–2531 (1996).
39. H. R. Costantino, R. Langer, and A. M. Klibanov. Moisture-induced aggregation of lyophilized insulin. *Pharm. Res.* **11**:21–29 (1994).
40. A. Shrake and P. D. Ross. Biphasic denaturation of human albumin due to ligand redistribution during unfolding. *J. Biol. Chem.* **263**:15392–15399 (1988).
41. H. M. Rawel, J. Kroll, and S. Rohn. Reactions of phenolic substances with lysozyme—Physicochemical characterisation and proteolytic digestion of the derivatives. *Food Chem.* **72**: 59–71 (2001).
42. W. S. Pierpoint. o-Quinones formed in plant extracts. Their reaction with bovine serum albumin. *Biochem. J.* **112**:619–629 (1969).
43. F. A. Carey. Phenols. In K. Emry and D. A. Damstra (eds.), *Organic Chemistry*, McGraw-Hill, New York, 1992, pp. 978–1011.
44. P. S. Brzovic, W. E. Choi, D. Borchardt, N. C. Kaarsholm, and M. F. Dunn. Structural asymmetry and half-site reactivity in the T to R allosteric transition of the insulin hexamer. *Biochemistry* **33**:13057–13069 (1994).
45. D. Ferrari, J. R. Diers, D. F. Bocian, N. C. Kaarsholm, and M. F. Dunn. Raman signatures of ligand binding and allosteric conformation change in hexameric insulin. *Biopolymers* **62**:249–260 (2001).
46. J. L. Whittingham, S. Chaudhuri, E. J. Dodson, P. C. Moody, and G. G. Dodson. X-ray crystallographic studies on hexameric insulins in the presence of helix-stabilizing agents, thiocyanate, methylparaben, and phenol. *Biochemistry* **34**:15553–15563 (1995).
47. E. Ciszak and G. D. Smith. Crystallographic evidence for dual coordination around zinc in the T3R3 human insulin hexamer. *Biochemistry* **33**:1512–1517 (1994).