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## *Biochimica et Biophysica Acta*

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Biochimica et Biophysica Acta 1208 (1994) 101–103

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Lori A. Hardaway <sup>a</sup>, David N. Brems <sup>b</sup>, John M. Beals <sup>b</sup>, Neil E. MacKenzie <sup>a,\*</sup>

<sup>a</sup> Department of Pharmaceutical Sciences, University of Arizona, Tucson, AZ 85721, USA

<sup>b</sup> Lilly Research Laboratories, Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285, USA

Received 14 December 1993; revised 18 April 1994



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**BIOCHIMICA ET BIOPHYSICA ACTA**  
International Journal of Biochemistry and Biophysics  
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Printed in The Netherlands

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## Amide hydrogen exchange of the central B-chain helix within the T- and R-states of insulin hexamers

Lori A. Hardaway<sup>a</sup>, David N. Brems<sup>b</sup>, John M. Beals<sup>b</sup>, Neil E. MacKenzie<sup>a,\*</sup>

<sup>a</sup> Department of Pharmaceutical Sciences, University of Arizona, Tucson, AZ 85721, USA

<sup>b</sup> Lilly Research Laboratories, Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285, USA

Received 14 December 1993; revised 18 April 1994

### Abstract

Comparative analysis of the <sup>1</sup>H-NMR spectra of human insulin shows that in the presence of the allosteric ligand, phenol, the tertiary structure of the protein is altered as evidenced by the decreased rate of amide hydrogen–deuterium exchange. In particular, exchange of amide protons in residues of the B-chain helix (B<sup>9</sup>–B<sup>20</sup>) are significantly affected suggesting either a stabilization of this helix or a reduction in the solvent accessibility of the helix in the R-state. This paper exemplifies the exchange rates of two amides (Val<sup>B18</sup> and Tyr<sup>B16</sup>) from this helix which decrease by approximately 400-fold as a result of this ligand induced conformational transition.

**Keywords:** Insulin; Excipient; NMR, <sup>1</sup>H-; Amide exchange; Allosteric conformation

### 1. Introduction

Insulin has been a protein of paramount importance since its discovery in 1921. Three insulin hexamers, T<sub>6</sub> [1], T<sub>3</sub>R<sub>3</sub> [2], and R<sub>6</sub> [3] have been described by X-ray crystallography and various solution-state spectral methods [4]. All hexamers exhibit a hydrophobic core composed of three  $\alpha$ -helices, two contained in the A chain (residues A<sup>1</sup>–A<sup>7</sup> and A<sup>14</sup>–A<sup>20</sup>) and the third in the B chain from residues B<sup>9</sup>–B<sup>20</sup>. The R<sub>6</sub> hexamer, which is induced by the presence of phenolic preservatives, has increased helical content in the N-terminus of the B chain (residues B<sup>1</sup>–B<sup>9</sup>), extending the helix from residues B<sup>1</sup>–B<sup>20</sup>, in contrast to the T<sub>6</sub> hexamer in which this region assumes an extended conformation. The T- and R-nomenclature of Monod [5] was applied by Dunn [6] to insulin in order to emphasize the allosteric conformational properties of the hexamer. The R-state is more compact, less flexible, and

the zinc exchange is retarded compared to the T-state. [3,4]. In addition, the R-state of insulin has been shown to be chemically more stable [7].

NMR spectroscopy studies require insulin in the monomeric state, presenting a considerable challenge given the proteins propensity to aggregate. The recent report of NMR studies on this protein under conditions in which the insulin monomer can be maintained at millimolar concentrations have been determined and an extensive number of the insulin hydrogen resonances have been assigned [8,9].

The thrust of this study is to determine by NMR spectroscopy the structural influence of phenol, a compound which is commonly used as an antimicrobial agent in pharmaceutical parenteral preparations. Thus the ability to identify and quantitate the stabilizing effects of excipient-induced conformational changes in insulin are of profound interest to the pharmaceutical industry and the development of insulin formulations that are chemically and physically more stable. As an example of this, we present the amide hydrogen–deuterium exchange rates for the Val<sup>B18</sup> and Tyr<sup>B16</sup> residues in the T<sub>6</sub> and R<sub>6</sub> human insulin hexamer.

### 2. Materials and methods

Zinc-free recombinant derived human insulin was obtained from Eli Lilly (lot number 214JK1). Deuterium

Abbreviations: NMR, Nuclear Magnetic Resonance Spectroscopy; 1D, one dimensional, T<sub>6</sub>, the crystalline two-zinc hexamer; T<sub>3</sub>R<sub>3</sub>, the crystalline hexamer species formed in the presence of SCN<sup>-</sup> or other members of the lyotropic anion series (Cl<sup>-</sup>, Br<sup>-</sup>, or I<sup>-</sup>); R<sub>6</sub>, the crystalline hexamer species formed in the presence of phenol; Tris, Tris(hydroxymethyl)aminomethane. Insulin amino-acid positions are denoted by the standard three-letter amino-acid code followed in superscript by its location in the A- or B-chain sequence position.

\* Corresponding author. Fax: +1 (602) 6264063.

oxide (99.9%), deuterated acetonitrile (99.96%) and trifluoroacetic acid were obtained from Cambridge Isotopes and used without further purification.

In order to study the amide exchange rates of the insulin hexamers with the provision that NMR spectral acquisition be performed on the monomer, the following method was developed. Human insulin was solubilized in Tris buffer and water in the presence of zinc, in the absence or presence of phenol to induce the formation of the  $T_6$  and  $R_6$  hexamers respectively. Solutions contained insulin (30 mg/ml), Tris buffer (50 mM), zinc (2 moles per insulin hexamer), and 50 mM phenol if present. This latter concentration is comparable to that found in parenteral formulations. Aliquots of these hexamer containing solutions were diluted 20-fold with solutions of deuterium oxide, Tris buffer (50 mM) at pH 8, and 50 mM phenol if present and allowed to exchange for varying periods of time. Insulin exchange occurs at 1.5 mg/ml, a concentration at which only the hexamer form is present [10].

After allowing exchange for varying periods of time, the amide exchange reaction was then quenched by lowering the pH to 3.0 [11]. The excipients were separated from insulin using a Sephadex G-25M column at 4°C, frozen and lyophilized. The time from sample quench to freezing for all samples was 12 min.

Samples were prepared for NMR analysis by reconstitution in  $D_2O$  (65%), deuterated acetonitrile (35%) and a small amount of trifluoroacetic acid to adjust the pH to 3.30–3.10 (pH meter reading) [8], placed in the spectrometer and shimmed. The time from sample reconstitution to data acquisition was maintained at 12 min for all samples.  $^1H$ -NMR spectra were acquired on a Bruker AM500 spectrometer, using a spectral width of 5000 Hz and an acquisition time of 16 min (264 scans plus 4 dummy scans).

Spectra were processed on a Bruker x32 work station using UNIX software (Bruker Instruments). Prior to Fourier transformation the data were multiplied by 0.5 Hz line broadening. Peak areas were calculated by a user-defined peaklist spectral deconvolution program. Rate constants were calculated by exponential least-squares analysis of plots of  $I = A_0 e^{-k_m t}$ , where  $I$  is the measured peak area,  $A_0$  is the initial peak area,  $k_m$  is the exchange rate constant ( $\text{min}^{-1}$ ) and  $t$  is time (min).

### 3. Results and discussion

The one-dimensional  $^1H$ -NMR spectra of the amide region of insulin at zero-time of exposure to deuterated non-phenolic and phenolic formulations (the  $T_6$  and  $R_6$  hexamer, respectively) are identical; the spectrum of the insulin monomer representing exchange from the  $T_6$  hexamer is shown in Fig. 1A. Assignments were made based on published data [8]. The amide resonances of Val<sup>B18</sup> and Tyr<sup>B16</sup> (V and Y respectively, Figure 1A) exhibit minimal overlap and this paper will focus on these two residues.

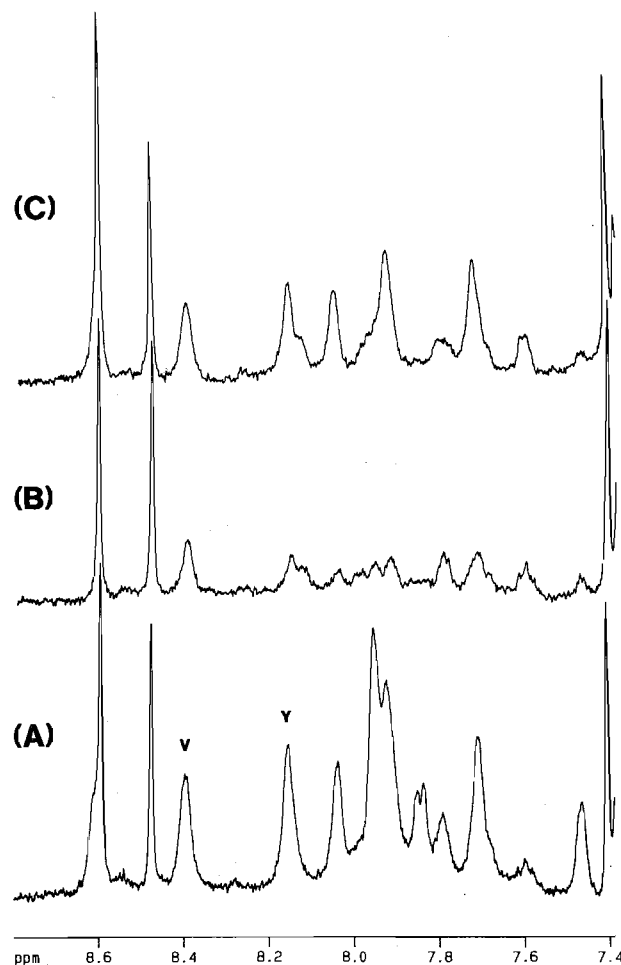


Fig. 1.  $^1H$ -NMR spectra of the amide region of insulin at zero-time (A), following 30 min of exchange in non-phenolic formulation (B), and following 24 h of exchange in phenolic formulation (C). Amide resonances of Val<sup>B18</sup> and Tyr<sup>B16</sup> are labelled as V and Y, respectively. The two sharp downfield resonances are the C2H resonances of His<sup>B10</sup> and His<sup>B5</sup>, respectively.

After 30 min of isotopic exchange, spectra representing exchange from the  $T_6$  hexamer (Fig. 1B) shows significant loss of signal intensity for all peaks. However, the spectra representing exchange from the  $R_6$  hexamer at this time (not shown) is virtually identical to that at zero-time. In fact, the spectra attributable to the  $R_6$  hexamer do not exhibit significant decrease in signal intensity until approx. 24 h (Fig. 1C). Following two weeks of exposure to deuterated formulation in the  $R_6$  hexamer, many amide resonance peaks are still recognizable (not shown). At the later time points, a resonance under Tyr<sup>B16</sup> is seen. This residue exhibits very slow exchange as it is approx. > 30% occupied following three weeks of exchange. The exchange rate of resonance Tyr<sup>B16</sup> was calculated independently of the effects of this underlying resonance.

The peak areas were calculated and plotted as a function of time. Plots for residues Val<sup>B18</sup> and Tyr<sup>B16</sup> in non-phenolic (Fig. 2) and phenolic (Fig. 3) formulation illustrates the marked difference in exchange rates. Al-

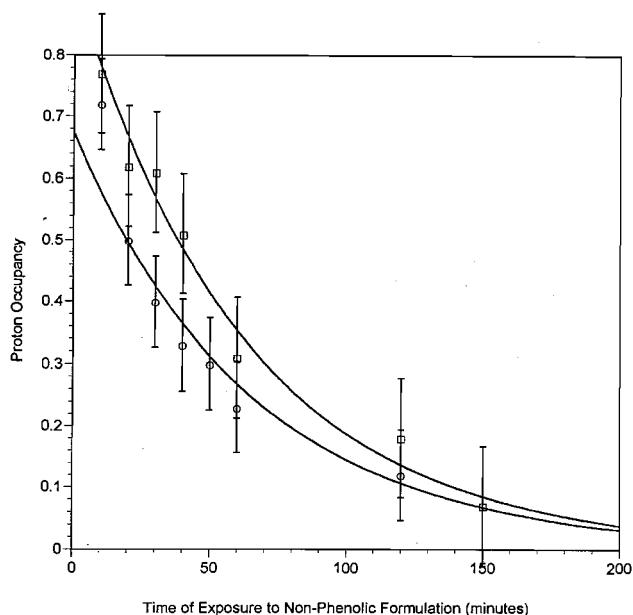


Fig. 2. Proton occupancy of the amide resonance of Val<sup>B18</sup> (□) and Tyr<sup>B16</sup> (○) vs. time (in minutes) of exposure to deuterated non-phenolic formulation.

though the data have a modest error, clearly proton occupancy decays as an exponential function. The amide exchange rate for Val<sup>B18</sup> in non-phenolic formulation is  $1.61 \cdot 10^{-2} \text{ min}^{-1}$  ( $R^2 = 0.97$ ) and in phenolic formulation is  $3.95 \cdot 10^{-5} \text{ min}^{-1}$  ( $R^2 = 0.97$ ). For Tyr<sup>B16</sup> in non-phenolic formulation the amide exchange rate is  $1.70 \cdot 10^{-2} \text{ min}^{-1}$  ( $R^2 = 0.93$ ) and in the phenolic formulation is  $3.82 \cdot 10^{-5} \text{ min}^{-1}$  ( $R^2 = 0.97$ ). Thus, in the presence of

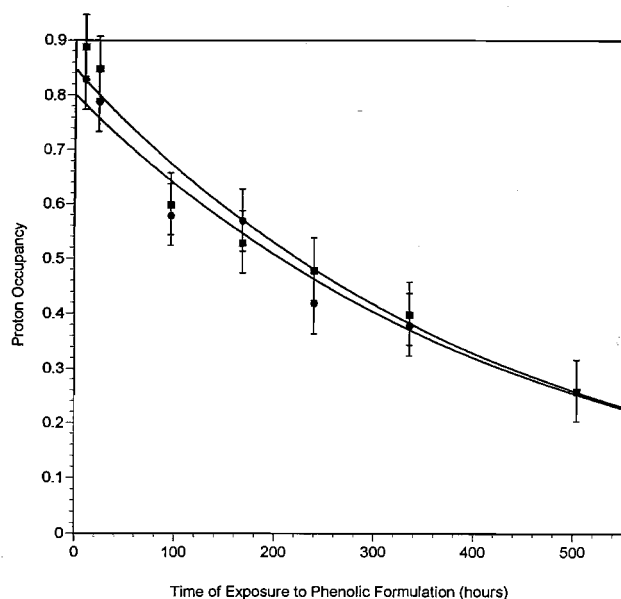


Fig. 3. Proton occupancy of the amide resonance of Val<sup>B18</sup> (■) and Tyr<sup>B16</sup> (●) vs. time (in hours) of exposure to deuterated phenolic formulation.

phenol, the amide exchange rate is slowed by approx. 400-fold. This decrease in exchange rate from the R-state may be due to either a reduction in the solvent accessibility of the B-chain helix in the R-state and/or an increase in the stability of the B-chain helix.

In the transition of the insulin hexamer into the R<sub>6</sub> structure, the T<sub>6</sub> hexamer binds phenol in each of six hydrophobic pockets that are formed by neighboring subunits and the two zinc ions become more deeply buried into the hexamer [3,4]. Thus, the R-state becomes more compact making the hexameric core less solvent accessible and protecting the B-chain helix amides from solvent exchange.

A second suggestion for the disparity of amide exchange rates between the two conformations may result from the presence of an increased helical region (B<sup>1</sup>–B<sup>9</sup>) to that of the extant helix (B<sup>9</sup>–B<sup>20</sup>). It may be that the resulting extended helix is more stable and therefore more resistant to amide exchange regardless of solvent exposure.

Computer modeling studies of the solvent accessibility of residues Val<sup>B18</sup> and Tyr<sup>B16</sup>, in the crystallographic determined structures of T<sub>6</sub> and R<sub>6</sub>, should yield a clear insight into an explanation of the observed reduced exchange rates.

This methodology now provides a tool for examining the effects of excipients, mutations, and environmental conditions on the conformation and stability of the insulin hexamer. The power of this methodology lies in the fact that effects can now be assessed at the molecular level.

## Acknowledgments

We thank Dr. Kathy McGovern and Constantine Job for valuable discussions and guidance.

## References

- [1] Adams, M.J., Baker, E.N., Blundell, T.L., Harding, M.M., Dodson, E.J., Hodgkin, D.D., Dodson, G.G., Rimmer, B., Vijayan, M. and Sheats, S. (1969) *Nature* 224, 491–495.
- [2] Bentley, G., Dodson, E.J., Dodson, G.G., Hodgkin, D.D., Mercola, D. (1976) *Nature* 261, 166–168.
- [3] Derewenda, U., Derewenda, Z., Dodson, E.J., Dodson, G.G., Reynolds, C.D., Smith, G.D., Sparks, C. and Swenson, D. (1989) *Nature* 338, 594–596.
- [4] Kaarsholm, N.C., Ko, H.-C. and Dunn, M.F. (1989) *Biochemistry* 28, 4427–4435.
- [5] Monod, J., Wyman, J. and Changeux, J.P. (1965) *J. Mol. Biol.* 12, 88–118.
- [6] Brader, M.L. and Dunn, M.F. (1991) *TIBS* 16, 341–345.
- [7] Brange, J. and Langkjær, L. (1992) *Acta Pharm. Nord.* 4, 149–158.
- [8] Kline, A.D. and Justice, R.M. (1990) *Biochemistry* 29, 2906–2913.
- [9] Hua, Q. and Weiss, M.A. (1991) *Biochemistry* 30, 5505–5515.
- [10] Brange, J. (1987) in *Galénics of Insulin*, Springer, New York.
- [11] Englander, S.W. and Kallenbach N.R. (1984) *Q. Rev. Biophys.* 16, 521–655.

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