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Direct Evidence of Conformational Heterogeneity in Human Pancreatic Glucokinase from High-Resolution Nuclear Magnetic Resonance†

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ABSTRACT: High-resolution nuclear magnetic resonance is used to investigate the conformational dynamics of human glucokinase, a 52 kDa monomeric enzyme that displays kinetic cooperativity. $^{1}H-^{15}N$ transverse relaxation optimized spectra of uniformly labeled glucokinase, recorded in the absence and presence of glucose, reveal significant cross-peak overlap and heterogeneous peak intensities that persist over a range of temperatures. ^{15}N-specific labeling of isoleucines and tryptophans, reporting on backbone and side chain dynamics, respectively, demonstrates that both unliganded and glucose-bound enzymes sample multiple conformations, although glucose stabilizes certain conformations. These results provide the first direct evidence of glucokinase conformational heterogeneity and hence shed light on the molecular basis of cooperativity.

Glucokinase is a 52 kDa enzyme that catalyzes the rate-limiting step of glucose metabolism in pancreatic β-cells (*1*, *2*). Glucokinase activity is allosterically regulated by its sugar substrate, displaying a sigmoidal response to glucose concentrations spanning the physiological range. The kinetic cooperativity of glucokinase is mechanistically interesting because the enzyme functions exclusively as a monomer (*3*). Current models suggest that cooperativity relies upon a slow, glucose-dependent conformational change that occurs with a rate constant slower than k_{cat} (*4*). Consistent with that postulate, crystal structures of glucokinase confirm that large-scale structural alterations accompany substrate binding (*5*). To date, essentially no experimental work has been directed toward understanding the functional dynamics of the enzyme. For this reason, we initiated an investigation of the solution state dynamics of glucokinase using high-resolution nuclear magnetic resonance (NMR).[1]

Initially, we recorded the $^{1}H-^{15}N$ TROSY spectrum of uniformly labeled glucokinase (120 μM) at 25 °C using an 800 MHz NMR spectrometer equipped with a cryogenic probe. This spectrum displayed very weak peak intensities and considerable cross-peak overlap. Increasing protein concentrations up to 520 μM did not remove heterogeneous peak intensities or alleviate cross-peak overlap. Analytical ultracentrifugation experiments at 40 μM indicated the presence of a monomeric species. One-dimensional ^{1}H and two-dimensional $^{1}H-^{15}N$ HSQC NMR spectra recorded over a range from 50 to 500 μM showed no change in the spectral characteristics with the increase in protein concentration (Figures S1 and S2 of the Supporting Information). Together, these data support retention of a monomeric state and preclude aggregation as the cause of the poor spectral resolution. Next, we recorded $^{1}H-^{15}N$ TROSY spectra over a range of temperatures from 5 to 45 °C. Above 40 °C, the protein rapidly precipitated. Below this temperature, we observed a consistent increase in spectral resolution and peak intensities with increasing temperatures (Figure S3 of the Supporting Information). Despite this fact, the 40 °C $^{1}H-^{15}N$ TROSY spectrum still displayed substantial cross-peak overlap and a heterogeneous distribution of peak intensities (Figure 1A). The size of glucokinase (52 kDa) does not fully explain its unusual NMR behavior, as indicated by comparison with the $^{1}H-^{15}N$ TROSY spectrum of monomeric arginine kinase (42 kDa), which displays a much weaker temperature dependence than glucokinase and a consistently higher spectral resolution (Figure S3 of the Supporting Information) (*6*). Interestingly, the addition of glucose to glucokinase did not alleviate cross-peak overlap, especially in the ^{1}H region near 8 ppm, although several new cross-peaks appeared (Figure 1B). Together, these observations suggest that glucokinase is an intrinsically mobile enzyme whose structure and dynamics are modulated by temperature and substrate binding.

To probe glucokinase dynamics via a different approach, we used NMR to detect the exchange of amide protons with D_2O. We initiated H−D exchange by dissolving a lyophilized sample of uniformly ^{15}N-labeled enzyme in 100% D_2O. Successive 2D $^{1}H-^{15}N$ HSQC spectra were recorded over a period of 20 h at pH 7.0 and 25 °C (Figure S4 of the Supporting Information). The $^{1}H-^{15}N$ HSQC spectra show a substantial loss of signal within the first 2 h of exchange. During this period, approximately 75% of the total signal was lost upon comparison to the 1D spectrum in H_2O. The rapidity of this loss prevented us from determining the effect of addition of glucose on the exchange rate of these protons. The remaining signal (~25%) underwent slower exchange, the rate of which was unaffected by the presence of glucose. The observation that a significant fraction of the amide protons undergoes relatively rapid exchange suggests that the backbone of one or more glucokinase conformational states is highly solvent exposed, both in the absence and in the presence of glucose.

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[1]Abbreviations: NMR, nuclear magnetic resonance; TROSY, transverse relaxation optimized spectroscopy; HSQC, heteronuclear single-quantum coherence spectroscopy; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TCEP, tris(2-carboxyethyl)phosphine.

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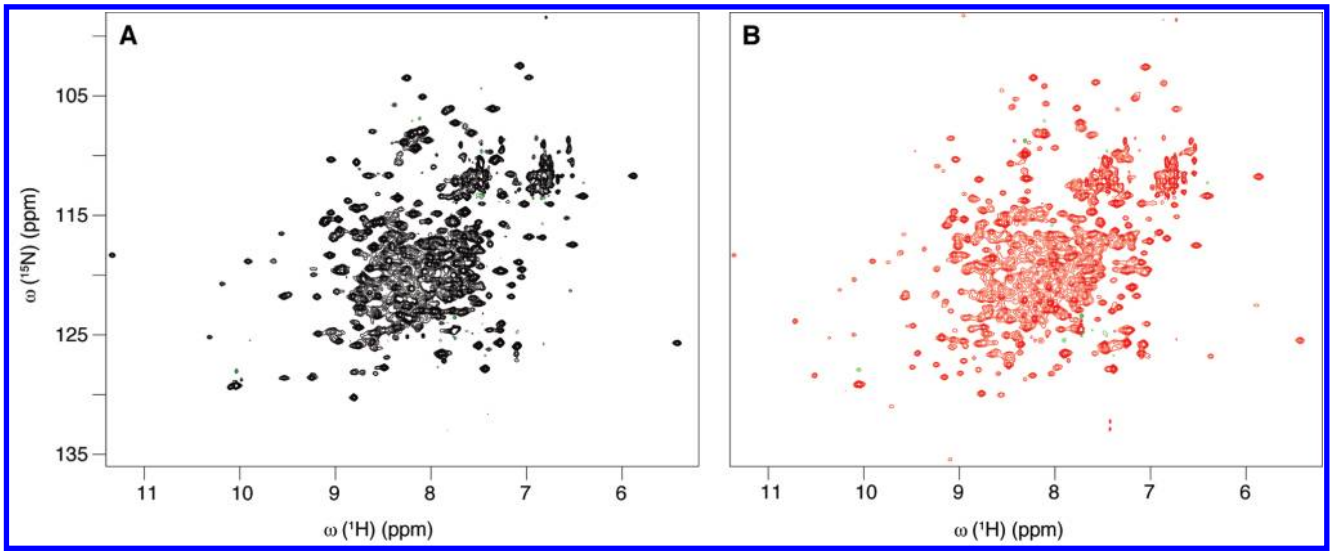

FIGURE 1: $^{1}H-^{15}N$ TROSY NMR spectrum at 800 MHz of uniformly ^{15}N-labeled glucokinase [520 μM in 50 mM HEPES (pH 7.0), 50 mM KCl, and 4 mM TCEP] in the absence (A) and presence (B) of 50 mM glucose. The spectra were recorded at 40 °C, as matrices of 2048 (N_2) × 290 (N_1) real points via accumulation of 512 scans (in the absence of glucose) or 128 scans (in the presence of glucose), multiplied by a cosine apodization function and zero-filled up to 2048 (N_2) × 512 (N_1) data points. Negative peaks (green) are due to the leakage of anti-TROSY contributions.

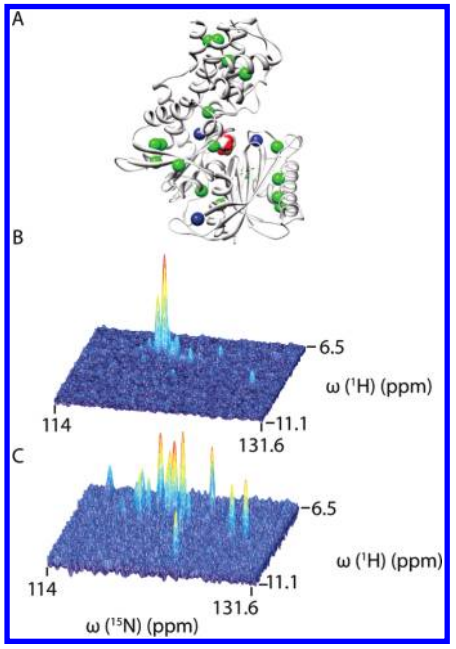

FIGURE 2: (A) Crystal structure of human glucokinase depicting the location of isoleucine (green spheres) and tryptophan (blue spheres) residues. Glucose is colored red and white. Mesh plots of $^{1}H-^{15}N$ TROSY spectra of [^{15}N]isoleucine-labeled glucokinase in the absence (B) and presence (C) of glucose, recorded at 40 °C. The image was created with Chimera (*10*) and Protein Data Bank entry 1V4S (*5*). For the sake of clarity, the allosteric activator is not depicted in the glucokinase structure.

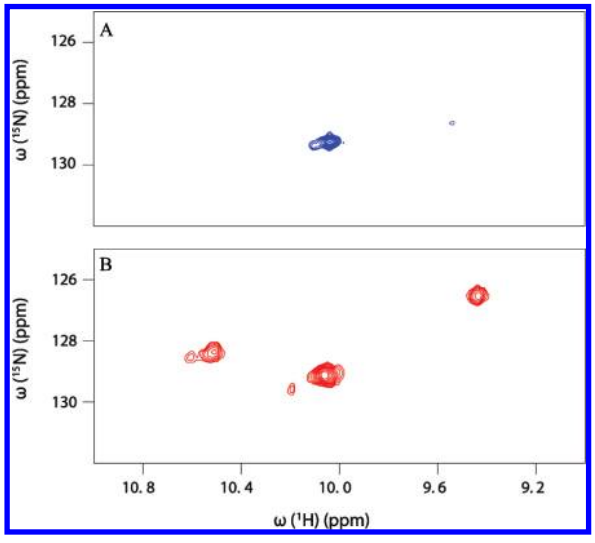

FIGURE 3: $^{1}H-^{15}N$ HSQC NMR spectra of glucokinase with [^{15}N]tryptophan side chain labels in the absence (A) and presence (B) of glucose. The spectra were recorded as matrices of 2048 (N_2) × 128 (N_1) or 2048 (N_2) × 64 (N_1) real points via accumulation of 128 or 64 scans in the absence or presence of glucose, respectively. The indirect dimension of the HSQC spectrum of the glucose-bound sample was extended up to 128 points by linear prediction. All dimensions were multiplied by a cosine square apodization function and zero filled up to 2048 (N_2) × 256 (N_1) data points. The spectrum in the absence of glucose was recorded using a smaller spectral window in the ^{15}N dimension (813 Hz compared to 2433 Hz in the presence of glucose).

To simplify the $^{1}H-^{15}N$ TROSY spectrum, and to detect discrete conformational states, we incorporated site-specific ^{15}N labels into the backbone atoms of isoleucine residues and the side chain atoms of tryptophan. Glucokinase contains 17 isoleucine residues that are intrinsically dispersed throughout the structure and have the potential to globally probe functional dynamics (Figure 2A). Glucokinase contains three tryptophan residues, two of which appear to experience significant environmental changes upon glucose association (*5*). Isoleucine and tryptophan labeling was achieved as previously described (*7*, *8*), and site-specific ^{15}N incorporation was confirmed via MALDI-TOF mass spectrometry (Figure S5 of the Supporting Information). The $^{1}H-^{15}N$ TROSY spectrum of unliganded, isoleucine-labeled glucokinase shows two strong cross-peaks and eight additional peaks that slightly exceed the noise level (Figure 2B). The addition of glucose leads to the emergence of extra cross-peaks and produces more homogeneous peak intensities (Figure 2C and Figure S6 of the Supporting Information). Similarly, the $^{1}H-^{15}N$ HSQC spectrum of unliganded, tryptophan-labeled glucokinase displays one strong cross-peak with a ^{1}H chemical shift at 10 ppm and a second, very weak cross-peak near 9.5 ppm (Figure 3A). In the presence of a saturating level of glucose, the $^{1}H-^{15}N$ HSQC

spectrum displays three prominent tryptophan cross-peaks, two of which possess weak extra peaks in their vicinity (Figure 3B). The differential number of cross-peaks observed in the absence and presence of glucose in both the isoleucine and tryptophan ^{15}N-labeled spectra demonstrates that glucose binding stabilizes distinct enzyme conformations. Moreover, the appearance of extra tryptophan side chain signals in the ^{1}H−^{15}N HSQC spectrum suggests that glucose-bound glucokinase samples multiple conformational states on a relatively slow time scale.

Several possible explanations exist for the unique spectral characteristics of human glucokinase. The differential number of cross-peaks observed in the absence and presence of glucose could be interpreted as unliganded glucokinase populating a large number of states. The resonances of these states would have to display a distribution of chemical shifts with peak intensities that are below the detection limit. From the signal-to-noise ratio of the tryptophan-labeled ^{1}H−^{15}N HSQC spectrum, we estimate an upper population limit for each of these states of ∼2%. A second possibility is that unliganded glucokinase undergoes conformational exchange between a smaller number of discrete states on a time scale that approaches the inverse of the chemical shift difference. The strong temperature dependence of the ^{1}H−^{15}N TROSY spectrum favors such a coalescence phenomenon (Figure S3 of the Supporting Information). A third possibility, very rapid amide proton exchange with the solvent, is unlikely to be responsible for cross-peak disappearance; even in the ^{1}H−^{15}N HSQC spectra of small highly flexible peptides, cross-peaks can be observed (*9*).

In summary, the results of our preliminary NMR investigation suggest that glucokinase is capable of sampling multiple conformational states, both in the absence and in the presence of glucose. The detection of additional cross-peaks in the glucose-bound spectrum suggests that glucose binding alters the dynamics of glucokinase conformational exchange. Indeed, the ability of glucose to modulate enzyme dynamics may provide a general mechanism by which this substrate can confer allosteric regulation. Using the sample conditions established here, quantitative NMR investigations to establish the rate of glucokinase conformational exchange in the presence of various cooperative and noncooperative ligands seem feasible. Such information is critical to our understanding of the link between enzyme functional dynamics and kinetic cooperativity.

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SUPPORTING INFORMATION AVAILABLE

Detailed labeling approaches and experimental procedures and Figures S1−S6. This material is available free of charge via the Internet at http://pubs.acs.org.

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