Structural instability of mutant β-cell glucokinase: implications for the molecular pathogenesis of maturity-onset diabetes of the young (type-2)

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The catalytic function and thermal stability of wild-type and mutant recombinant human pancreatic β-cell glucokinase was investigated. The mutants E70K and E300K, which are thought to be the cause of impaired insulin production by the pancreatic β-cell and decreased glucose uptake by the liver of patients with maturity-onset diabetes of the young, were found to be functionally indistinguishable from the wild-type, i.e. their k_{cat}, $S_{0.5}$, inflection point and h were normal. However, these two mutants showed markedly reduced stability under a variety of test conditions. Glucokinase instability, not low enzyme catalytic activity, may be the cause of diabetes mellitus with E70K and E300K mutants.

INTRODUCTION

Glucokinase (GK), a high-K_m hexokinase, plays a central role in glucose homoeostasis by serving as the glucose sensor in pancreatic β-cells and by controlling glucose metabolism in the liver [1–6]. It might also be involved in glucose sensing by certain other neural and neuroendocrine cell types. The basic concept of the GK glucose sensor has a sound theoretical and solid experimental basis and was convincingly reaffirmed by the recent discovery of GK gene mutations in families with an early-onset dominantly inherited non-insulin-dependent diabetes mellitus termed maturity-onset diabetes of the young type II (MODY-2). Over 30 specific different mutations of the GK gene have since been reported [1,7–11]. The effects of these mutations on function of the enzyme have been determined and compared with those of wild-type GK [7–11]. These initial surveys revealed that the k_{cat}, $S_{0.5}$ (the latter frequently referred to as apparent glucose k_m) and the Hill coefficient (h) for glucose as substrate of the mutant GKs were altered such that diminished catalytic activity might explain the MODY-2 phenotype. However, the catalytic defects reported for some mutants were small and the marginal change in enzyme activity did not seem to parallel the clinical manifestations [10], suggesting that diminished protein stability may be another cause of enzyme deficiency in this disease [11].

Previously we reported that introduction of the E300K mutation into the human islet GK isoform significantly increased thermal instability of the enzyme when compared with the wild-type [9,11]. However, interpretation of this result was uncertain because the k_{cat} of recombinant enzyme preparations was also reduced by about 50 %, which could be sufficient to explain the diabetic phenotype without invoking enzyme instability as a crucial factor. By using a rapid purification scheme, we now show that E70K and E300K are virtually indistinguishable from the wild-type with respect to their k_{cat}, $S_{0.5}$, h and inflection point but that both show striking thermal instability. These results indicate that structural instability of a catalytically normal GK might be a cause of diabetes in MODY-2 families with E70K, E300K, and by extrapolation may play a role in other GK mutations.

EXPERIMENTAL

Recombinant human wild-type and mutant β-cell glutathione S-transferase (GST)–GK was prepared as previously described [9] and the enzyme was stored at −80 °C in 50 mM Tris/HCl buffer, pH 8.0, containing 200 mM KCl, 10 mM GSH, 5 mM dithiothreitol, 30 % glycerol and 50 mM glucose. The purified recombinant GKs were screened for purity by SDS/PAGE [9]. GK activity was measured spectrophotometrically as described with some modification [9]. The assay reagent contained 100 mM Tris/HCl, pH 7.4, 6 mM $MgCl_2$, 14 mM mercaptoethanol, 0.1 % BSA, 150 mM KCl, 5 mM ATP, 0.4 mM $NADP^+$, 2.5 units/ml glucose-6-phosphate dehydrogenase and a sufficient number of suitable concentrations of glucose to allow the determinations of $S_{0.5}$ for glucose and h. All data sets were fitted to an equation of the form

$$v = \frac{V_{max}[\text{glucose}]}{K_m + [\text{glucose}]}$$

by plotting [glucose]/v against [glucose] and fitting the linear portion of the curve with a straight line (where the x intercept is $-K_m$ and the slope is V_{max}). The V_{max} data were normalized by dividing the mol of enzyme used in the assay which is the definition of k_{cat}[$k_{cat} = V_{max}$/(mol of GK)]. As the concentration-dependence curve is sigmoidal, there was a systematic misfit between the data and the model fit. If the plateau region of the curve is well characterized, then the resulting error from this will be an underestimation of the concentration at which the velocity is half-maximal. However, the V_{max} and k_{cat} will be estimated within a 10–15 % error as shown by comparison between two different methods of data analysis (Table 1). This was adequate

Abbreviations used: MODY-2, maturity-onset diabetes of the young; GK, glucokinase; GST–GK, glutathione S-transferase–glucokinase.

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Table 1 Kinetic constants of human recombinant wild-type and MODY-2 mutant β-cell GST–GK fusion protein

The results from 16 preparations are recorded. Means ± S.E.M. are given. k_{cat} values refer to turnover at 30 °C. The subscripts H and M refer to the methods of data analysis, i.e. non-linear and linear optimization. The $S_{0.5}$ value was obtained from the Hill equation and K_m (app) from the Hanes–Woolf transformation of the Michaelis–Menten equation.

Preparation	Protein yield (mg/l)	GK stock (mg/ml)	k_{cat_H} (s^{-1})	k_{cat_M} (s^{-1})	$S_{0.5}$ (mM)	K_m (mM)	h_H	h_M	Inflection point (mM)	$k_{cat_H}/S_{0.5}$	k_{cat_M}/K_m
Wild-type											
1	18.00	0.90	87.54	93.75	9.31	6.93	1.82	1.44	4.71	9.40	13.53
2	17.60	0.80	82.15	87.50	8.43	6.25	1.80	1.41	4.20	9.74	14.00
3	20.00	1.00	84.36	89.36	7.12	5.23	1.74	1.43	3.35	11.85	17.09
4	11.20	0.64	85.29	91.07	7.96	6.07	1.75	1.41	3.78	10.71	15.00
Mean ± S.E.M.	16.60 ± 2.20	0.84 ± 0.09	84.84 ± 1.35	90.42 ± 1.56	8.21 ± 0.55	6.12 ± 0.43	1.78 ± 0.02	1.42 ± 0.01	4.01 ± 0.34	10.43 ± 0.61	14.91 ± 0.89
E70K											
5	3.20	0.50	102.38	109.06	9.84	7.40	1.72	1.43	4.52	10.40	14.74
6	1.80	0.26	86.52	92.70	9.54	7.05	1.81	1.33	4.80	9.07	13.15
7	0.80	0.10	29.47	29.65	8.50	7.04	1.18	1.30	1.03	3.47	4.21
8	4.90	0.64	100.68	107.39	10.50	8.13	1.67	1.47	4.58	9.59	13.21
Mean ± S.E.M.	2.70 ± 1.03	0.38 ± 0.14	79.76 ± 18.23	84.70 ± 19.85	9.59 ± 0.50	7.40 ± 0.27	1.60 ± 0.16	1.38 ± 0.04	3.73 ± 0.89	8.13 ± 1.73	11.33 ± 2.63
E300K											
9	2.30	0.46	78.32	85.10	10.36	8.18	1.89	1.46	5.55	7.56	10.40
10	0.60	0.09	46.50	50.08	9.30	7.59	1.66	1.33	4.05	5.00	6.60
11	1.60	0.27	79.40	86.57	10.87	9.28	1.76	1.40	5.23	7.30	9.33
12	1.80	0.26	73.16	79.29	10.54	8.81	1.72	1.42	4.86	6.94	9.00
13	3.00	0.37	89.45	96.06	9.90	7.87	1.73	1.43	4.57	9.04	12.21
Mean ± S.E.M.	1.90 ± 0.48	0.29 ± 0.07	73.37 ± 8.59	79.42 ± 9.20	10.19 ± 0.31	8.35 ± 0.34	1.75 ± 0.05	1.41 ± 0.03	4.85 ± 0.30	7.17 ± 0.81	9.51 ± 1.12
V203A											
14	11.90	1.38	25.70	43.67	50.69	123.47	1.69	1.48	22.66	0.40	0.26
15	9.30	1.16	25.50	49.70	63.02	188.82	1.50	1.39	23.40	0.51	0.35
16	5.80	0.76	31.05	58.00	55.86	154.91	1.72	1.42	25.79	0.56	0.37
Mean ± S.E.M.	9.00 ± 2.03	1.10 ± 0.21	27.42 ± 1.85	50.46 ± 4.78	56.52 ± 4.11	155.73 ± 21.78	1.64 ± 0.07	1.43 ± 0.03	23.95 ± 1.04	0.49 ± 0.05	0.33 ± 0.04

when relative changes in k_{cat} were being examined in the stability tests. In order to obtain an accurate measure of the half-maximal glucose concentration ($S_{0.5}$) and a precise measure of the sigmoidicity, in addition to the V_{max}, the concentration-dependence curves were also fitted to a Hill equation of the form

$$v = \frac{V_{max}[\text{glucose}]^h}{S_{0.5}{}^h + [\text{glucose}]^h}$$

where v is the velocity of the reaction in mol/s, V_{max} is the maximal velocity, $S_{0.5}$ is the concentration at which the velocity is half-maximal (in mM) and h is the Hill coefficient which does not have to be an integer and is unitless. The data fitting was carried out using a non-linear optimization program contained in Kaleidagraph (Abelbeck Software, Malvern, PA, U.S.A.) which is based on the Levenberg–Marquardt algorithm [12]. The concentration of glucose at which the inflection point occurs was calculated as described by Segel [13]:

$$[\text{glucose}]_{IP} = S_{0.5}\left(\frac{h-1}{h+1}\right)^{\frac{1}{h}}$$

Escherichia coli XL-1 Blue was obtained from Stratagene. Restriction enzymes were purchased from Gibco–BRL. *Taq* DNA polymerase and dNTPs for PCR were from Perkin–Elmer. ATP, $NADP^+$ and glucose-6-phosphate dehydrogenase were from Boehringer-Mannheim Biochemicals. Glutathione–agarose beads and other chemicals were obtained from Sigma.

RESULTS

Purity and kinetic characteristics of recombinant human wild-type and mutant GKs

Wild-type and mutant GST–GK obtained after elution from a glutathione–agarose column were virtually pure in each case (Figure 1) with a single band at 75 kDa representing the GK fusion protein. Determination of the k_{cat_M}, k_{cat_H}, K_m(app), $S_{0.5}$, h and the inflection point showed that E70K, E300K and wild-type enzymes were virtually indistinguishable (Figure 2 and Table 1), although there was a trend towards slightly higher $S_{0.5}$ and K_m(app) values for the E70K and E300K mutants. The V203A mutation was used as an additional reference molecule because previous studies suggested it to be a low-activity mutant with both a low k_{cat} and a high K_m(app) or $S_{0.5}$ [8,9]. We found here that the k_{cat30} of V203A was about one-third of that observed in the wild-type, and the glucose affinity of this mutant was much lower than the control value, in agreement with earlier observations [1,8,9]. The Hill coefficients of all preparations were comparable. It is noteworthy that the yield of two mutants was consistently lower than the yield of the wild-type preparation, 16 % and 11 % of the wild-type for E70K and E300K respectively. In contrast, the yield of V203A was similar to that of the wild-type enzyme. The data presented in Table 1 illustrate that the uncritical application of the Michaelis–Menten equation to estimate kinetic constants for GK could lead to erroneous results, most strikingly an overestimation of the affinity of GK for glucose. The error is quantitatively quite substantial in the case of the mutant V203A. In this case the V_{max} is off by 50 % and the affinity by 3-fold. Figure 2 shows, however, that the Hanes–Woolf plot (S versus S/V) is very helpful for documenting the marginal non-linearity of the glucose-dependence of the enzyme. It is noteworthy that extrapolation from this linear analysis results in a lower h than the non-linear optimization.

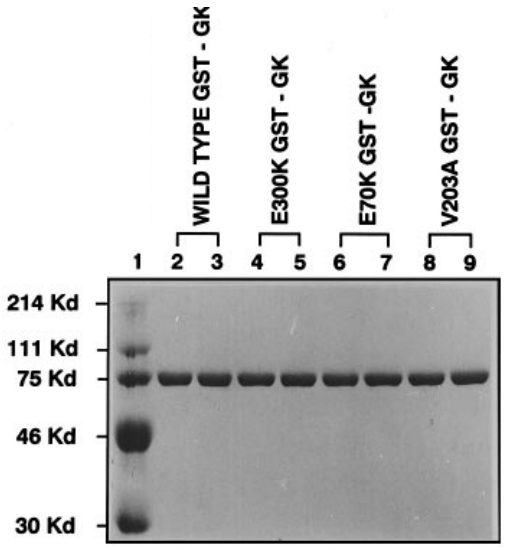

Figure 1 One-step purification of the β-cell isoform of human GST–GK by the method of Liang et al. [9]

Protein samples (4 μg each) of two recombinant preparations each of wild-type and mutants E70K, E300K and V203A were separated by SDS/PAGE. The fusion protein showed the expected mass of 75 kDa assessed by comparison with molecular-mass standards in lane 1 (given in kDa).

Thermal stability of recombinant human wild-type and mutant GKs

Analysis of the thermal stability of GST–GKs (Figure 3) showed that the wild-type enzyme was slightly activated after 30 min incubation at 47.5, 50 and 52.5 °C compared with the results at 0, 30, 40 and 45 °C, but its activity fell abruptly to about 50 % at 55 °C and was practically undetectable at 60 °C. This activation between 47.5 and 52.5 °C was highly reproducible and was observed in all three wild-type preparations that were examined in detail. In contrast, the three mutants showed much greater thermal lability than the wild-type enzyme with a fall of activity to one-half or less at 52.5 °C, a further decline at 55 °C and complete loss at 60 °C. E70K and V203A also did not show, and E300K barely exhibited, the small increase in k_{cat30} after pre-incubation at 45–52.5 °C. Since the differences in stability between wild-type and mutants were most striking between 50 and 60 °C, a time course of thermal decay was performed at 55 °C, which showed again a clear difference in lability of the four enzymes (Figure 4). Under these conditions the wild-type was relatively stable with no change during the first 5 min of the test but a 50 % loss of activity after 30 min. The mutants decayed in a manner that was not strictly first-order. Half the activity of E70K was lost after about 8 min, and, after 30 min at 55 °C, 18 % of activity still remained. The $S_{0.5}$ had increased slightly such that it was about 50 % higher than that of the wild-type under comparable conditions (not shown). Interestingly, V203A appeared to be even more unstable. Half of its activity was lost after 2–3 min at 55 °C and after 30 min it was practically inactive. E300K behaved similarly to E70K, with half of its activity lost at 7.5 min and about 10 % remaining at 30 min. The $S_{0.5}$ of E300K had increased by about 50 %, apparently higher than the $S_{0.5}$ of the wild-type under comparable conditions (not shown). The Hill coefficients of the enzymes were not influenced by the same treatment.

To obtain a more precise measure of differences in thermal stability between wild-type and mutant enzymes, detailed tem-

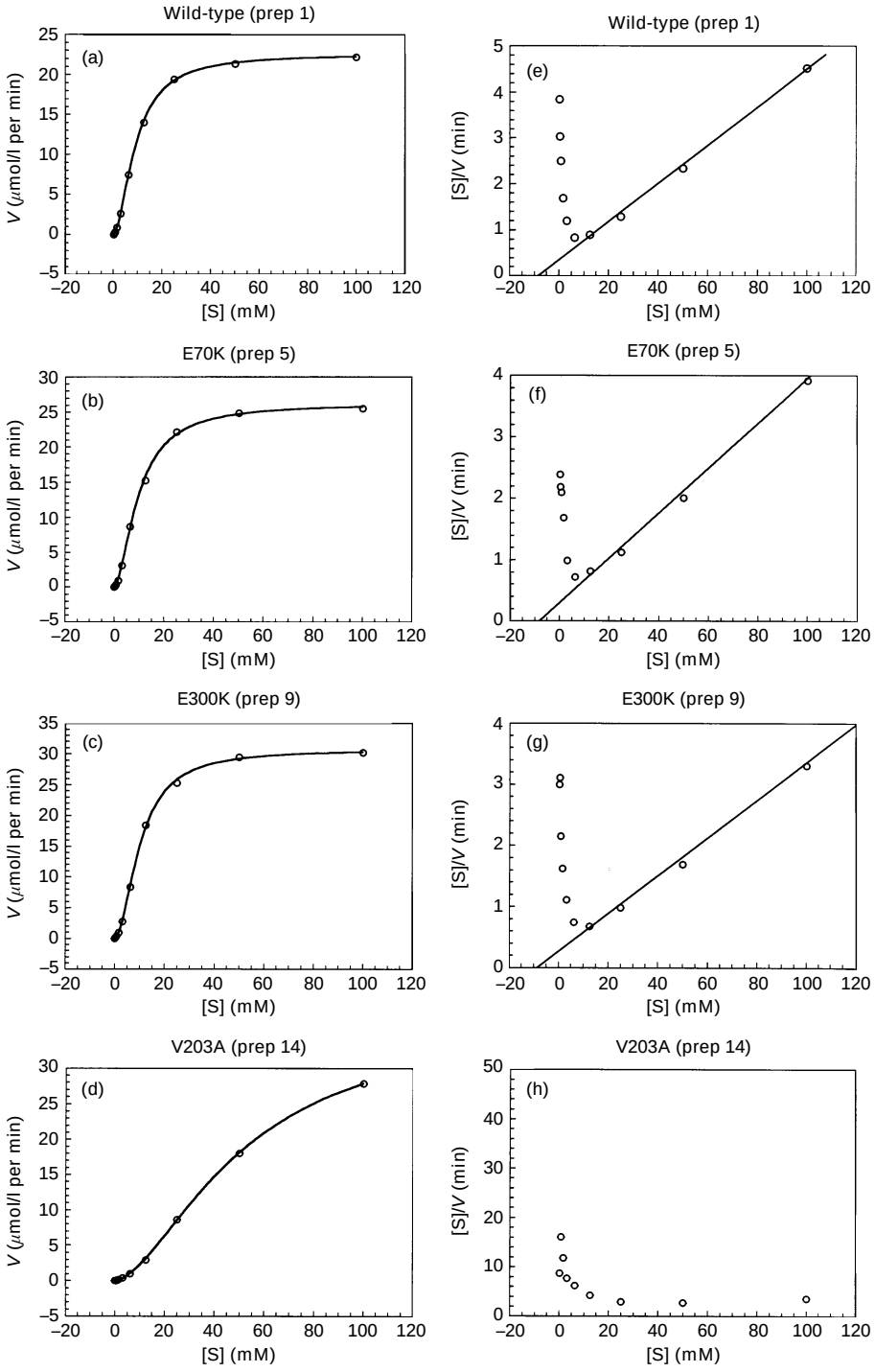

Figure 2 Comparison of non-linear optimization using the Hill equation (a–d) with the Hanes–Woolf transformation of the Michaelis–Menten equation (e–h)

(**a**)–(**d**) show perfect fits, whereas fitting the Hanes–Woolf plot is clearly problematical (**e**–**h**). This is particularly obvious for V203A as seen in (**h**). The protein concentrations in the assays were as follows: preps 1 and 5, 0.32 μg/ml; prep 9, 0.49 μg/ml; prep 14, 1.78 μg/ml. The assays were performed at 30 °C.

perature titration studies were performed (Figure 5). With glucose at 15 mM and using about 100 μg/ml recombinant purified protein, it was seen that GST–GK was extremely sensitive to very small changes in temperature. The wild-type enzyme retained full activity for 90 min at 45 °C (Figure 5A) whereas both E70K and E300K were rapidly destroyed at 41 °C (Figures 5B and 5D). V203A was intermediate in its stability.

Lastly, the instability of the mutants was tested under conditions which closely resembled *in vivo* conditions by performing a stability test at 37 °C (Figure 6). At 37 °C and with the

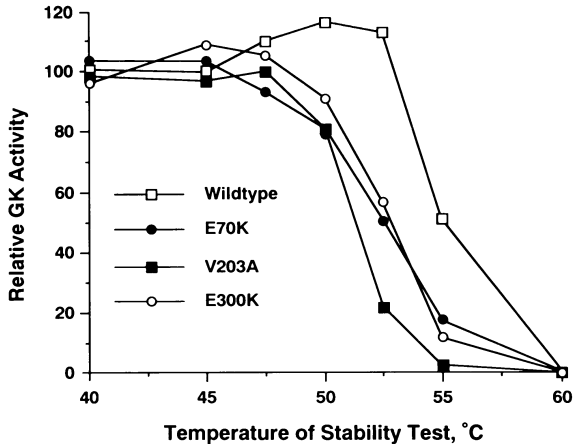

Figure 3 Effect of temperature on wild-type mutant recombinant β-cell GST–GK fusion protein

The stability test was carried out in storage buffer as mentioned in the text with 50 mM glucose and 30% glycerol present. The enzyme was incubated for 30 min at different temperatures ranging from 30 to 60 °C and then assayed over an observation period of 5 min at 30 °C in the recording spectrophotometer. Three experiments were performed for each case: wild-type (preparations 1, 2 and 4 of Table 1 using an average protein concentration of 300 μg/ml); E70K (preparations 5, 6 and 8 of Table 1 using an average protein concentration of 465 μg/ml); V203A (preparations 14, 15 and 16 of Table 1 using 300 μg/ml protein on average) and E300K (preparations 9, 11 and 12 at 287 μg/ml protein on average). Means of three experiments are shown for each case. Reproducibility was high and S.E.M.s are therefore not included for the sake of clarity.

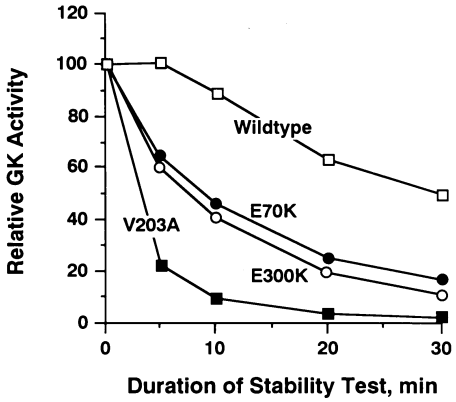

Figure 4 Stability of wild-type and mutant recombinant human β-cell GK at 55 °C

The enzyme was incubated for different lengths of time from 5 to 30 min at 55 °C with 50 mM glucose and 30% glycerol in the incubation buffer. Three experiments were performed in each case and the means are shown. The S.E.M.s are not included for the sake of clarity. The average protein concentrations were 300, 465, 300 and 287 μg/ml for wild-type, E70K, V203A and E300K respectively.

concentration of the stabilizing molecules glucose, K^+, glycerol, mercaptoethanol and dithiothreitol reduced to one-third of the concentration of the storage buffer, the stability difference between wild-type and mutants was absolute rather than relative. The decay curves of E70K and E300K were nearly superimposable, and the rate of decline appeared to slow down with time such that it deviated from first-order kinetics, approaching around 10% of starting level after 30 min. There was again a trend toward an increase in $S_{0.5}$ values as these two mutants decayed (not shown). V203A lost activity more slowly at this temperature with about 70% remaining after 30 min.

DISCUSSION

Reduced GK activity in cells that express the enzyme, specifically pancreatic β-cells, hepatocytes and probably others, provides a plausible biochemical explanation for the development of MODY-2 in patients with GK gene mutations [1–11]. The present data indicate that the loss of GK catalytic activity in non-insulin-dependent diabetes mellitus caused by either an E70K or E300K mutation are due to protein instability rather than an intrinsically low k_{cat}. V203A can be classified as a low-activity mutant because its k_{cat} and $S_{0.5}$ are markedly changed compared with the wild-type, but the abnormal enzyme appears also to be less stable than the wild-type as observed under all experimental conditions of this study (Figures 3–6). The GK deficit of pancreatic B-cells resulting from mutations that cause low stability (E70K and E300K) or low activity combined with low stability (V203A) would be expected to cause decreased insulin release [1,2,10,11]. The postulated loss of hepatic GK activity is probably due to two factors. First, there would be reduced induction of GK biosynthesis because insulin is diminished. Second, there would be a diminished half-life of the enzyme. This interpretation is based on extrapolations to the two described human liver isoforms from the present results with the β-cell isoform of GK. These extrapolations are reasonable because all GK isoforms are indistinguishable kinetically [9]. These predictions are also verifiable with recombinant liver enzyme and using sensitive assays that would allow reliable quantification of the enzyme activity in less than 100 cells of biopsy samples [14,15].

The E70K and E300K GK gene mutations and their corresponding amino acid substitutions in the protein lead to dramatic charge changes at two different locations based on the predicted structure of GK derived from the X-ray structure of crystalline yeast hexokinase B [8]. At physiological pH, glutamate is negatively charged and lysine is positively charged such that critical salt bridges involving E70 and E300 might be interrupted when lysine is substituted. The effect of this loss of critical charge interaction might be impaired folding during biosynthesis and more rapid unfolding and denaturation of the mature enzyme at physiological temperatures. The low expression in *E. coli* and the thermal instability as shown here are probably a manifestation of these mutations. This hypothesis can be tested further by substituting aspartate for glutamate and arginine for lysine with the prediction that the resulting enzyme mutant will be virtually indistinguishable from the wild-type and the spontaneous mutants respectively.

Previous studies failed to recognize that the E70K and E300K mutants are catalytically normal [1,7–9]. Instead they indicated that the k_{cat} was decreased and the glucose K_m(app) or $S_{0.5}$ was found to be normal or increased. It is likely that increased speed and efficiency of purification made possible by the GST fusion protein paradigm and affinity chromatography may have contributed to the different results of the present work. Previously we have shown that attachment of a 25 kDa N-terminal fusion protein partner to GK does not adversely affect any of the kinetic properties of the enzyme including the k_{cat}, K_m(app) for glucose and ATP, the K_i of long-chain acyl-CoAs, h and indices of hysteresis [9,11]. The differences between these and previous studies suggest that multistep purification schemes should be used with caution when protein stability is in question. Although these classical procedures yield the native product, they might

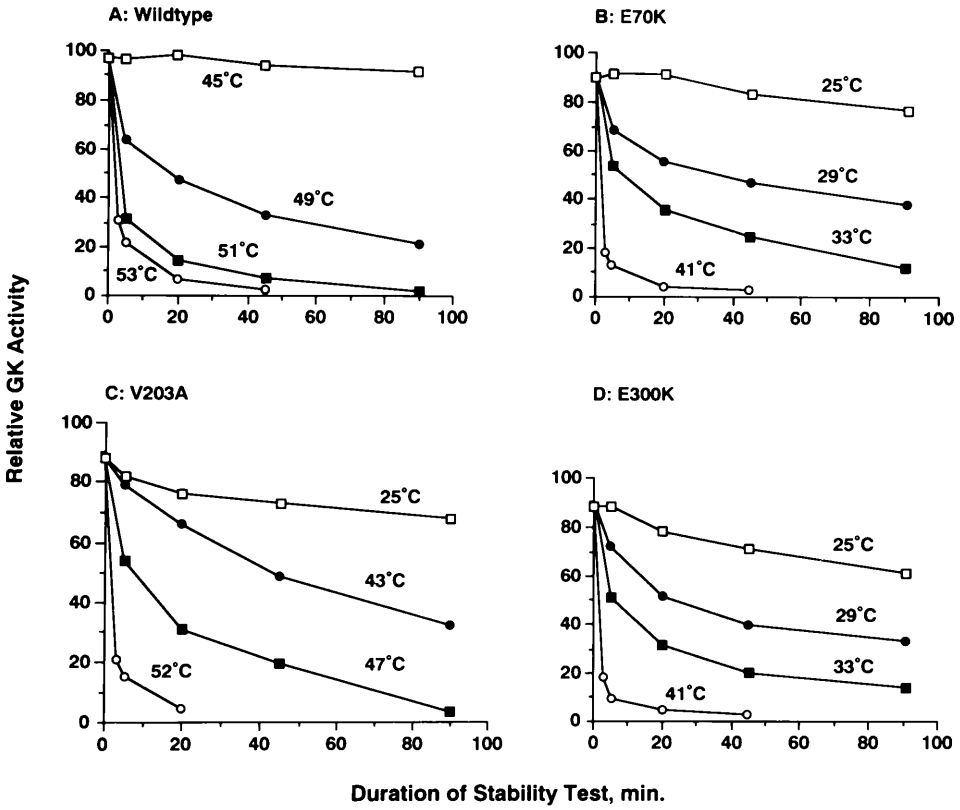

Figure 5 Temperature titration of wild-type and mutant human GK

The enzyme was incubated in one-third-strength storage buffer at different temperatures ranging from 25 to 53 °C for different lengths of time from 3 to 90 min and then assayed spectrophotometrically at 30 °C. Glucose concentration was 15 mM and glycerol 10%. (**A**) Wild-type preparations 1, 2 and 3 (shown in Table 1) were used at an average protein level of 100 μg/ml; (**B**) E70K preparations 5, 6 and 8 were used at an average protein level of 118 μg/ml; (**C**) V203A preparations 14, 15, 16 were used at an average protein concentration of 100 μg/ml; (**D**) E300K preparations 9, 11 and 13 were used at 114 μg/ml on average. The means are presented.

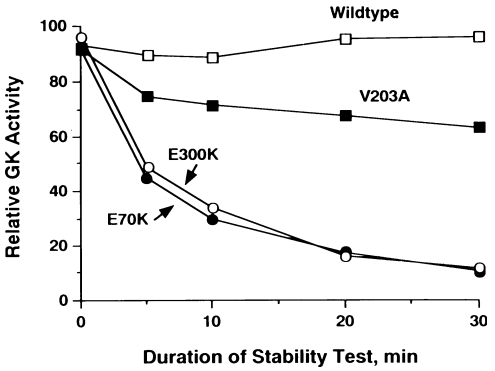

Figure 6 Stability of wild-type and mutant β-cell GK under physiological conditions

A comparison was made of the stability of wild-type and mutant enzymes at 37 °C during incubation in one-third-strength storage buffer with 15 mM glucose and 10% glycerol present followed by the routine spectrometric assay. Three or four experiments were performed for each case. The means of these experiments are recorded. Wild-type preparations 1, 2 and 3 at 100 μg/ml protein were used; E70K preparations 5, 6 and 8 were used at 96 μg/ml protein; V203A preparations 14, 15 and 16 were used at 90 μg/ml protein; E300K preparations 9, 11, 12 and 13 were used at 90 μg/ml protein.

not be suitable for use with unstable mutants. In contrast, the use of a fusion protein appears to be advantageous for stability analysis of labile mutants.

The present data including a comparison of two methods of analysis (linear analysis according to classical Michaelis–Menten formulations and non-linear optimization using the Hill equation) illustrate that it is mandatory to use non-linear optimization techniques in the case of the sigmoidal enzyme GK (Table 1, Figure 2) as convincingly argued by Luz-Cardenas in her recent monograph [5]. In many instances the error might be small, as exemplified by mutants E70K and E300K, but in other cases the error might be very significant as is true for V203A. Application of the non-linear optimization in analysing the kinetic data using the Hill equation resulted also in higher $S_{0.5}$ values for glucose (8.2 compared with 6.1 mM) and h (1.8 compared with 1.4) when compared with the results of an analysis using the Michaelis–Menten equation. These differences may seem small at first glance but, in view of the crucial role of GK as pacemaker of glycolysis and glucose sensor of the pancreatic β-cells, they are probably of biological significance. A glucose-phosphorylating enzyme with an $S_{0.5}$ for glucose of about 8.0, h of 1.8 and an inflection point of about 4.0 appears to be ideally designed to serve as a glucose sensor.

The discovery of instability mutants of GK underscores the potential importance that enzyme stability might play in the physiological regulation of GK activity in B-cells and hepatocytes. High glucose induces the enzyme mass and activity in β-cells under many conditions and insulin enhances biosynthesis of GK in liver cells [1,8,16]. It seems that, in the β-cell, regulation of GK expression is post-transcriptional and might be due to alteration of enzyme stability by the glucose molecule itself

[1,11]. This view is strengthened because glucose has a dramatic effect on GK stability and decay and protects the enzyme against toxins such as alloxan [17,18]. The relative instability of V203A is probably a consequence of its abnormally high $S_{0.5}$ for glucose mitigating the physiological protection of the enzyme by its substrate. Thus other diabetogenic mutant GKs with a high $S_{0.5}$ for glucose might also be relatively unstable when the ambient glucose is about 10 mM. S131P, G175R, V182M, A188T, E256K, E279Q and K414E are examples [7–9]. Instability mutants suggest a strategy for exploring the possibility of pharmacological intervention to stabilize the enzyme. This might be possible through the substrate-binding site or through an allosteric site of the enzyme which has been demonstrated to bind long-chain acyl-CoA or hexose-6-phosphate-dependent regulatory protein [19]. The results also raise the question about the physiological mechanism of GK protein degradation and the possibility that the affinity of the enzyme for glucose may change as it undergoes structural changes since the $S_{0.5}$ seems to increase as the enzyme decays. Since the catalytic function of GK is drastically altered by changes in critical thiol groups, future stability studies need also to consider this aspect [17,18]. It has recently been proposed that GK may exist in a bound and free form [3,20]. It is also logical to ask whether immobilization of the enzyme, by whatever means and to whatever matrix of the cell, might alter its thermal lability and its kinetic features. The low-stability mutants E70K and E300K offer attractive models for the exploration of such lines of speculation.

Evidence for the existence of instability mutants of GK as a cause for diabetes extends what is known about human biochemical genetics [21]. Instability of proteins is a well-established factor in the pathogenesis of anaemias resulting from classical cases of mutations in the haemoglobin and glucose-6-phosphate dehydrogenase genes [21] and may play a role in many other diseases [22]. The present results are the first significant step to show that this fundamental mechanism might also affect GK thereby causing diabetes.

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