Hexokinases catalyse the ATP-dependent phosphorylation of hexoses, with glucose being the most common substrate. This represents the first enzymic step in metabolism of this hexose by a number of pathways. Since metabolism of glucose, particularly through glycolysis, is of fundamental importance in virtually all living organisms, hexokinases are of widespread occurrence. Comparison of amino acid sequences, deduced from the cloned cDNAs, makes it clear that these enzymes comprise a homologous family \([1-3]\) represented in microbes, plants and animals; this family also includes several kinases that use substrates other than hexoses (e.g. glycerol, xylulose and ribulose). Moreover, the hexokinase family is itself a subset of a larger class of proteins that share a common ATP-binding motif, dubbed the 'actin fold' \([4]\). Reference \([1]\) provides a general review of the hexokinases.

Four isoenzymes of hexokinase are found in mammalian tissues. The type-I, type-II and type-III isoenzymes \([5]\), also referred to as hexokinases A–C \([6]\) respectively, are similar in having a \(K_m\) for glucose in the submillimolar range, molecular masses of approx. 100 kDa and in their susceptibility to allosteric inhibition by the product, glucose 6-phosphate (G6P). In contrast, the type-IV isoenzyme (hexokinase D), commonly called glucokinase, has a \(K_m\) for glucose in the millimolar range, a molecular mass of 50 kDa and is not sensitive to inhibition by physiologically relevant levels of G6P; in these properties, the type-IV isoenzyme is similar to the hexokinases found in yeast. On the basis of these observations, Easterby and O'Brien \([7]\) and Colowick \([8]\) independently suggested that the 100 kDa mammalian isoenzymes types I–III might have evolved by duplication and fusion of a gene encoding an ancestral 50 kDa hexokinase resembling the present-day yeast hexokinase and mammalian type-IV isoenzymes. It was suggested that one of the duplicated catalytic sites had evolved into the allosteric site for G6P, resulting in the sensitivity to this inhibitor seen with the 100 kDa enzymes.

Work with the type-I isoenzyme indicated that catalytic function was associated solely with the C-terminal half of the molecule \([9-16]\), whereas regulatory function was associated with the N-terminal half \([12,17,18]\). Moreover, the N- and C-terminal halves of the mammalian type-I–III isoenzymes show extensive sequence similarity to both each other and 50 kDa hexokinases found in other organisms \([1,2,19]\). These results were consistent with the Colowick–Easterby proposal \([7,8]\). However, the finding that the isolated C-terminal (catalytic) half of type-I hexokinase remained sensitive to inhibition by G6P \([11]\) was not compatible with the proposed evolutionary scheme, leading to the suggestion \([11]\) that sensitivity to G6P arose before the gene duplication and fusion event giving rise to the 100 kDa mammalian isoenzymes. More recent work \([20,21]\) has demonstrated that, in contrast with the type-I isoenzyme, both the N- and C-terminal halves of the type-II isoenzyme possess G6P-sensitive catalytic activity. Thus the type-II isoenzyme most closely resembles the ancestral enzyme that would have been expected to result from duplication and fusion of a gene encoding an ancestral 50 kDa G6P-sensitive hexokinase; on this basis, it was suggested \([21]\) that the type-II isoenzyme is the more 'ancient' of the mammalian isoenzymes. Further duplication of the fused gene resulted in other 100 kDa iso-

**Abbreviation used:** G6P, glucose 6-phosphate.
enzymes, with additional mutations leading to decreased sequence similarity between the halves and to functional differentiation, as seen in the type-I isoenzyme. On the basis of the even greater divergence between the sequences of the N- and C-terminal halves of the type-III isoenzyme, functional differentiation of the N- and C-terminal halves of this isoenzyme was predicted [21]. Recent unpublished work with Henry Tsai has now indicated that, as with the type-I isoenzyme, catalytic function appears to be associated exclusively with the C-terminal half of the type-III isoenzyme; however, other aspects of the functional organization, e.g. whether G6P inhibition is dependent on binding to an allosteric site in the N-terminal half, as found in the type-I isoenzyme, remain to be determined. Our present view of the evolutionary relationships among the hexokinases is represented in Figure 1.

The scheme in Figure 1 may address the question of how the 100 kDa mammalian isoenzymes came to be, but a different question, asked many years ago by Tito Ureta [24], is ‘Why isoenzymes?’ The existence of isoenzymes presumably reflects some functional differences in the physiological context. If all we needed was an enzyme that could phosphorylate glucose, then our tissues would probably still have something like yeast hexokinase. If sensitivity to inhibition by G6P was important, then why couldn’t we get along with a 50 kDa G6P-sensitive enzyme, like starfish or S. mansoni? And even if there is some advantage in having a 100 kDa G6P-sensitive hexokinase, why do we need three distinct isoenzymes of this sort? The answers to such questions are likely to be complex. On the basis of present understanding of the mammalian isoenzymes, at least three factors come immediately to mind: differences in kinetic or regulatory properties, differences in subcellular location, and differences in transcriptional regulation. Although listed separately, it is not implied that these are unrelated, nor is it implied that these are the only factors that might be relevant in determining unique physiological roles for the isoenzymes. They do, however, provide a basis for further discussion of these isoenzymes.

There is considerable variation in the basic kinetic parameters reported by various investigators [25,26]. Nonetheless, it seems clear that there are substantial differences, probably orders of magnitude, in the apparent affinity of these isoenzymes for glucose, with the type-III enzyme having the lowest \( K_{\text{m}} \) and the type-II form the highest. In addition, type-III hexokinase shows substrate inhibition at glucose concentrations above about 0.5 mM, whereas this is not seen with the other isoenzymes. There are modest differences in \( K_{\text{m}} \) for ATP, and somewhat greater differences in sensitivity to inhibition by G6P, the type-III enzyme being the least

**Evolutionary relationships among the hexokinases**

Molecular masses, approx. 50 or 100 kDa, are indicated by lengths of the lines; the catalytic site is represented by a filled circle and an allosteric site for binding of the product inhibitor, G6P, by a filled square. One evolutionary path from an ancestral 50 kDa enzyme led to the 50 kDa G6P-insensitive hexokinases as found in present-day yeast. In an alternative pathway, sensitivity to inhibition by G6P was acquired; direct descendants of this ancestral 50 kDa G6P-sensitive hexokinase persist in contemporary organisms such as starfish and the parasitic blood fluke Schistosoma mansoni. Duplication and fusion of the gene encoding an ancestral 50 kDa G6P-sensitive hexokinase gave rise to a 100 kDa G6P-sensitive ‘mammalian’ hexokinase; the present-day type-II isoenzyme most closely resembles the 100 kDa enzyme expected to result from this duplication-fusion event. Further duplications of the gene encoding the 100 kDa hexokinase, and additional mutations leading to functional differentiation of the N- and C-terminal halves, gave rise to the type-I and type-III isoenzymes. The C-terminus of the type-I isoenzyme is thought to contain a latent binding site for G6P (open square) and the N-terminal half contains a latent binding site for glucose (open circle); see reference [1] or [11] for more extensive discussion of the functional organization of the type-I isoenzyme. Catalytic function has also been associated with the C-terminal half of the type-III isoenzyme (H. J. Tsai and J. E. Wilson, unpublished work) but other features of the functional organization of this isoenzyme remain to be determined. On the basis of amino acid sequence similarity, the 50 kDa type-IV isoenzyme is much more closely related to the 100 kDa mammalian isoenzymes than it is to the 50 kDa hexokinases found in non-mammalian organisms. Thus a re-splitting of the gene encoding a 100 kDa mammalian hexokinase is suggested [22,23] to have given rise to the type-IV isoenzyme (as opposed to the direct evolution of type-IV hexokinase from ancestral 50 kDa forms).
sensitive. The possible significance of these differences in affinities or the substrate inhibition of type-III hexokinase for function in vivo remains unclear.

Perhaps more significant are differences in response to P, [26,27]. Inhibition of type-I hexokinase by G6P is competitively antagonized by P, consistent with the mutually exclusive binding of these ligands [27]. While the debate continues on this issue, our view is that these ligands compete for a common binding site in the N-terminal half of the enzyme [12]. Henry Tsai has constructed chimaeric hexokinases and demonstrated that the ability of P, to antagonize inhibition by G6P transfers with the N-terminal half of type-I hexokinase when the latter is fused with the C-terminal half of the type-II isoenzyme in a chimaeric construct [28]. At higher concentrations, above about 3 mM or so, P, becomes an inhibitor of type-I hexokinase. This bimodal action of P, — antagonizing G6P inhibition at low concentrations then itself becoming inhibitory at higher concentrations — implies the existence of two distinct sites for P, and we believe that inhibition results from binding to a lower-affinity site in the C-terminal half of the molecule [11,12,28].

In contrast with the situation with the type-I isoenzyme, P, does not antagonize inhibition of type-II hexokinase by G6P but is, at all concentrations, itself an inhibitor. Thus, the type-I and type-II hexokinases are expected to respond differently to changes in the G6P/P, ratio. It seems reasonable to attribute physiological relevance to this difference [26]. The argument has been made that the type-I isoenzyme plays primarily a catabolic role, introducing blood-derived glucose into glycolytic metabolism with the ultimate objective being energy production. This is consistent with the ubiquitous expression of this isoenzyme, and with the very high levels found in brain, an organ well known for its dependence on blood glucose as a substrate for a very active energy metabolism. Levels of G6P and P, tend to vary inversely during periods of altered metabolic demand. Thus the regulatory properties of the type-I isoenzyme are ideally suited to respond appropriately to changes in energy status as reflected by the G6P/P, ratio.

Inhibition of type-II hexokinase by these ligands is additive, and thus this isoenzyme is expected to be most active under conditions in which intracellular levels of both G6P and P, are relatively low. It can be argued [26] that this favourably adapts the type-II isoenzyme to function as an anabolic enzyme, introducing glucose into metabolism leading to storage forms such as glycogen or fat. However, when endogenous glycogen stores have been depleted by exhaustive exercise [29] or chronic stimulation [30,31], leading to increased reliance on blood-borne glucose as a substrate, levels of type-II hexokinase are increased and this isoenzyme apparently has a primarily catabolic role.

Now all of this is too simplistic because it implies that hexokinase activity is merely responsive to changes in cellular metabolite levels, essentially following classical Michaelis-Menten kinetics. It is certainly more complicated than that, and distinct subcellular localization of the isoenzymes is likely to be a significant factor in determining their physiological roles.

In contrast with other glycolytic enzymes, substantial amounts of type-I hexokinase are found associated with the mitochondrial fraction in homogenates of various tissues [26,32-34], e.g. in brain, more than 80% of the enzyme may be particulate. The mitochondrial localization of the type-I isoenzyme has also been demonstrated by immunohistochemical methods [35-37]. There is considerable evidence to support the view that this physical relationship between hexokinase and mitochondria leads to intimate metabolic interactions, with the bound hexokinase preferentially, perhaps exclusively, using as substrate ATP produced by intramitochondrial oxidative phosphorylation [1,38]. The potential physiological significance of this has been discussed previously [1].

Type-II hexokinase may also bind to mitochondria [39,40] and have 'privileged access' to intramitochondrially produced ATP [41,42]. However, interpretation of the latter studies is complicated by the fact that significant amounts of the type-I isoenzyme were probably also present on the mitochondria.

In contrast with the type-I and type-II isoenzymes, the type-III enzyme has been found to be localized at the periphery of the nucleus [43,44], perhaps associated with the nuclear pore structures. The possible physiological consequences of this relationship remain unclear. It is, however, interesting to note that distinct isoenzymes — type I bound to mitochondria, and type III at the nuclear periphery — may coexist in the same cell [43,44], suggesting that these isoenzymes play discrete metabolic roles, i.e. that the metabolic fate of the G6P depends on which isoenzyme produces it, and where.
Finally, the existence of isoenzymes, encoded by discrete genes, may offer the possibility for differences in regulation of hexokinase activity by various physiologically relevant signals. There is, at present, virtually nothing known about the regulation of the expression of the type-III isoenzyme, a void that needs to be addressed in the future. However, it is certainly clear that expression of the type-I and type-II isoenzymes are regulated quite differently. Thus insulin induces increased transcription of type-II hexokinase but has no effect on expression of the type-I isoenzyme [45], and it appears that, in muscle, expression of the type-II isoenzyme is also selectively affected by exercise or chronic stimulation [29-31]. Understanding the transcriptional regulation of these genes requires characterization of the respective promoters. The promoter for the type-II isoenzyme has been isolated [46-48] and shown to contain classical TATA and CCAAT elements. In contrast, the promoter region for the type-I isoenzyme has been found to lack these elements, and to utilize multiple transcriptional start sites (J. White, W. Liu and J. E. Wilson, unpublished work); both promoter and transcriptional start sites are contained within a CpG-rich region, commonly referred to as a 'CpG island' [49]. The characteristics of the type-I promoter region are frequently associated with 'housekeeping genes' [49]; the ubiquitous expression of the type-I isoenzyme in mammalian tissues would certainly seem to qualify it for such a designation.

Phosphorylation of glucose is of fundamental importance in mammalian metabolism. The evolution of multiple forms of mammalian hexokinase has provided the possibility for sophisticated regulation of this reaction by classical allosteric mechanisms as well as compartmentation of G6P formation within the cell, with underlying transcriptional regulation governed by distinct physiological parameters.

The hexokinases (HKs), a family of closely related enzymes, catalyse the phosphorylation of hexose to produce hexose 6-phosphate. The four mammalian HKs (ATP-D-hexose 6-phosphotransferase; EC2.7.1.1) are designated I, II, III and IV. These enzymes, although highly conserved in amino acid sequence, differ in molecular mass, tissue distribution, regulation and catalytic properties. HKI–III have molecular masses of approx. 100 kDa, show a broad but distinct tissue distribution, have a relatively high affinity for glucose, and are subject to feedback regulation by physiological levels of glucose 6-phosphate (G6P) [1]. HKIV, more commonly referred to as glucokinase (GK), has a molecular mass of approx. 50 kDa, is primarily located in liver and pancreatic β-cells, has a lower affinity for glucose, and is not subject to feedback regulation by physiological levels of G6P [2]. The difference in molecular mass between GK and HKI–III led several investigators to postulate that the larger HKs (HKI–III) arose from a GK-like gene through gene duplication and tandem ligation [3–5]. A comparison of GK with the various HKs, based on direct and deduced amino acid sequence information, supports this hypothesis but does not provide information about how this occurred.

Glucose enters cells through a specific member of a family of glucose transporters, designated GLUT1–4 [6]. The phosphorylation of glucose by the HKs ensures glucose entry into cells through members of the glucose-transporter family via facilitated diffusion along a downhill concentration gradient, and thus HK provides the ‘pull’ for glucose entry into the cell. The tissue distribution of the various HK family members, intracellular location and relationship to glucose transporters, mechanism of action and regulation are all of interest. In this regard, we chose to focus our studies on the HK isoenzyme HKII and present here a summary of the information we have obtained about the structure of the gene, the regulation of the gene, the protein structure and the role of HKII in glucose metabolism.

Received 23 July 1996