Differences in Catalytic Properties between Cerebral Cytoplasmic and Mitochondrial Hexokinases

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1. Clear kinetic differences between cytoplasmic and mitochondrial forms of type-I cerebral hexokinase were demonstrated from experiments performed under identical conditions on three (cytoplasmic, bound mitochondrial and solubilized mitochondrial) preparations of the enzyme. 2. Whereas the Michaelis constant for glucose (K_m^{Glc}) was consistent, that for $MgATP^{2-}$ (K_m^{ATP}) was lower in the cytoplasmic than in the two mitochondrial preparations. The substrate dissociation constants (K_s^{Glc} and K_s^{ATP}) were both higher in the cytoplasmic than in the mitochondrial preparations. A further difference in the substrate kinetic patterns was that $K_m^{ATP} = K_s^{ATP}$ for the cytoplasmic enzyme, in contrast with the mitochondrial enzyme, where K_m^{ATP} was clearly not equal to K_s^{ATP} [Bachelard *et al.* (1971) *Biochem. J.* 123, 707–715]. 3. Dead-end inhibition produced by *N*-acetylglucosamine and by AMP also exhibited different quantitative kinetic patterns for the two enzyme sources. Both inhibitions gave K_i values similar or equal to those of K_i' for the cytoplasmic activity, whereas K_i was clearly not equal to K_i' for the mitochondrial activity. 4. All of these studies demonstrated the similarity of the two mitochondrial activities (particulate and solubilized), which were both clearly different from the cytoplasmic activity. 5. The analysis gives a practical example of our previous theoretical treatment on the derivation of true inhibition constants. 6. The results are discussed in terms of the function of cerebral hexokinases.

Brain hexokinase exists in essentially two sub-cellular sites: a particulate form, bound to the mitochondria, and a soluble cytoplasmic form (Stern, 1954; Johnson, 1960; Bachelard, 1967). Previous studies have shown no clear differences in the properties *in vitro* of the major constituent (type I) of the hexokinase activities of the cytoplasmic and mitochondrial fractions of the mammalian cerebral cortex. The evidence included similarity of molecular weight (Grossbard & Schimke, 1966), electrophoretic mobility (Bachelard, 1967; Bigl *et al.*, 1971), column-chromatographic behaviour (Thompson & Bachelard, 1970), and the more qualitative aspects of their kinetic properties and reaction mechanism (Bachelard *et al.*, 1971). These properties appeared to be similar in the three preparations examined (cytoplasmic, particulate mitochondrial and solubilized mitochondrial). Such results do not eliminate the possibility that the cytoplasmic activity may be an artifact of preparation (see Vallejo *et al.*, 1970) rather than possessing a separate identity and localization within the cells *in vivo*.

Alternatively there has been some speculation on the possibility that the cytoplasmic enzyme may be located in the glia whereas particulate hexokinase is located in the neurons (Bigl *et al.*, 1971; Kellogg *et al.*, 1974). However, there is no direct evidence by which these suggestions can be assessed.

A subsequent scrutiny of our published kinetic results (Bachelard *et al.*, 1971) led us to suspect that the quantitative differences noted between the type-I hexokinase activities of the major subcellular sources may have been significant. It became clear to us during an analysis of the theoretical derivation of K_i values (Thompson & Bachelard, 1976) that those which we had reported for dead-end inhibitors were apparent rather than true, and also that the slight quantitative differences observed in substrate kinetic constants might be real, rather than due to the experimental variation that we had assumed to be present.

True K_i values cannot be derived directly from any primary or secondary kinetic plot of dead-end inhibition where the reaction mechanism is rapid-equilibrium random (as shown for this enzyme; Bachelard *et al.*, 1971). The derived apparent K_i value is, in both competitive and non-competitive inhibition, a complex factor which includes substrate kinetic constants in addition to K_i (Thompson & Bachelard, 1976). We therefore re-examined our previous data

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(Bachelard *et al.*, 1971) and performed additional kinetic studies on the purified solubilized mitochondrial activity to complete the analysis. The results show experimental verification of the previous theoretical treatment (Thompson & Bachelard, 1976) and bring out clear quantitative differences in the catalytic properties between the cytoplasmic and mitochondrial activities.

Methods

Preparation of solubilized mitochondrial hexokinase

Ox cerebral-cortex mitochondria were prepared by sucrose-density-gradient centrifugation (Sottocasa *et al.*, 1967) and the type-I hexokinase activity was then extracted and purified essentially by the method of Redkar & Kenkare (1972). The final preparation had a specific activity of 110 $\mu\text{mol}/\text{min}$ per mg of protein at 37°C. Protein was measured by the method of Lowry *et al.* (1951) with crystalline bovine plasma albumin as standard.

Measurement of hexokinase activity

The conditions of enzymic assay were as described previously (Bachelard *et al.*, 1971) with the following variations. The concentration of Mg^{2+} was 6.75 mM total, 5.00 mM total or a constant calculated excess of 1.35 mM over the calculated MgATP^{2-} (Bachelard & Goldfarb, 1969). The anion species were either Cl^- or acetate; triethanolamine (free base) was adjusted to pH 7.6 with HCl or acetic acid and the final concentration was 100 mM. The Mg^{2+} salt added had the same anion species as the buffer in each case, as indicated in the text. The kinetic results are presented as weighted regression lines (Wilkinson, 1961). Secondary plots of slopes and intercepts of double-reciprocal plots were regression lines drawn as described by Florini & Vestling (1957).

Reagents

The sodium salts of ATP and NADP^+ , and glucose 6-phosphate dehydrogenase (EC 1.1.1.49, from yeast), were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. *N*-Acetylglucosamine and all other chemicals were of the highest purity available from BDH Chemicals, Poole, Dorset, U.K.

Symbols used

The symbols (see Table 1) are mainly those approved by the IUPAC-IUB Commission on Biochemical Nomenclature. To avoid confusion, the corresponding symbols used in Bachelard *et al.* (1971)

Table 1. *Symbols used*

K_m^{ATP}	Michaelis constant for MgATP (K_A ; Bachelard <i>et al.</i> , 1971).
K_s^{ATP}	Dissociation constant of E-MgATP (K_{AG}/K_G ; Bachelard <i>et al.</i> , 1971).
K_m^{Glc}	Michaelis constant for glucose (K_G ; Bachelard <i>et al.</i> , 1971).
K_s^{Glc}	Dissociation constant of E-glucose (K_{AG}/K_A ; Bachelard <i>et al.</i> , 1971).
K_i	Dissociation constant of enzyme-inhibitor complex (EI).
K_i'	Equilibrium constant of the dissociation of inhibitor from enzyme-substrate-inhibitor complex (ESI) (see Thompson & Bachelard, 1976).
K_i^{slopes}	Apparent K_i value obtained from secondary plots of slopes, taken from plots of $1/v$ versus $1/s$, versus appropriate [inhibitor] (K_{i1} ; Thompson & Bachelard, 1976).
$K_i^{\text{intercepts}}$	Apparent K_i values obtained from secondary plots of intercepts, taken from plots of $1/v$ versus $1/s$, versus appropriate [inhibitor] (K_{i1} ; Thompson & Bachelard, 1976).
K_s^A	Dissociation constant of $\text{EA} \rightleftharpoons \text{E} + \text{A}$, in the general case where A is the first substrate to bind to free enzyme, E. (K_{i1} ; Thompson & Bachelard, 1976).
K_m^A	Michaelis constant for first substrate, A, in the general case (K_s ; Thompson & Bachelard, 1976).
K_m^B	Michaelis constant for second substrate, B, in the general case (K_s ; Thompson & Bachelard, 1976). In the rapid-equilibrium random mechanism, either glucose or MgATP can be A.

and Thompson & Bachelard (1976) are included (in parentheses) where appropriate.

Results

Substrate kinetic analysis of solubilized mitochondrial hexokinase

Representative double-reciprocal plots made with respect to glucose and to MgATP^{2-} are presented in Figs. 1(a) and 1(b) respectively; the corresponding respective secondary plots of intercepts are Figs. 1(c) and 1(d). The common intersection points of Figs. 1(a) and 1(b) were not on the abscissa, in contrast with the patterns observed for the cytoplasmic activity, where the intersection points were clearly abscissal (Bachelard *et al.*, 1971). The abscissal co-ordinates gave the following results with Cl^- as anion: $K_s^{\text{Glc}} = 0.02 \text{ mM}$ (Fig. 1a), $K_s^{\text{ATP}} = 0.22 \text{ mM}$ (Fig. 1b), $K_m^{\text{ATP}} = 0.76 \text{ mM}$ (Fig. 1c) and $K_m^{\text{Glc}} = 0.06 \text{ mM}$ (Fig. 1d). The values presented in Table 2 are the means of the separate series of experiments performed, with the variations in Mg^{2+} concentration and anion species as shown.

Fig. 1. Substrate kinetic analysis of solubilized cerebral mitochondrial hexokinase

(a) Plot of $1/v$ versus $1/[\text{glucose}]$ with the following MgATP^{2-} concentrations: $\circ$, 0.133 mM; $\bullet$, 0.2 mM; $\square$, 0.33 mM; $\blacksquare$, 0.50 mM; $\triangle$, 1.0 mM; $\blacktriangle$, 2.0 mM. (b) Plot of $1/v$ versus $1/[\text{MgATP}^{2-}]$ with glucose concentrations: $\circ$, 0.015 mM; $\bullet$, 0.020 mM; $\triangle$, 0.033 mM; $\blacksquare$, 0.067 mM; $\square$, 0.20 mM. (c) Secondary plot of intercepts from (a) versus $1/[\text{MgATP}^{2-}]$. (d) Secondary plot of intercepts from (b) versus $1/[\text{glucose}]$. Experimental details are described in the text.

Table 2. *Substrate kinetic constants of purified solubilized mitochondrial hexokinase and partially purified cytoplasmic hexokinase*

Values were derived as described in the text. The Mg^{2+} concentrations were 6.75 mM (total), 5.0 mM (total) or a constant calculated excess of 1.35 mM over the calculated $MgATP^{2-}$ concentration. The anion species were either Cl^- or acetate.

Mg^{2+} (mM)	Anion	No. of expts.	K_m^{Glc} (mM)	K_m^{ATP} (mM)	K_s^{Glc} (mM)	K_s^{ATP*} (mM)	$K_s^{ATP†}$ (mM)
Mitochondrial enzyme							
5.0 (total)	Acetate	2	0.075	0.77	0.018	0.17	0.20
6.75 (total)	Cl^-	2	0.060	0.70	0.019	0.20	0.20
1.35 (constant excess)	Cl^-	1	0.066	0.69	0.018	0.24	0.21
Overall	Mean $\pm$ s.d.	5	0.066 ± 0.009	0.71 ± 0.09	0.018 ± 0.001	0.20 ± 0.034	0.20 ± 0.033
Cytoplasmic enzyme‡							
6.75 (total)	Cl^-	2	0.060	0.40	0.055	0.43	—

* K_s^{ATP} from common intersection point of double-reciprocal plot.

† K_s^{ATP} calculated from the abscissal intercept ($K_s^{ATP} K_m^{Glc} / K_m^{ATP}$) from secondary plot of slopes (of plot of $1/v$ versus $1/[MgATP^{2-}]$) versus $1/[glucose]$.

‡ Data from Bachelard *et al.* (1971) where $K_s^{Glc} = K_{AG}/K_A$; and $K_s^{ATP} = K_{AG}/K_G$.

Inhibition kinetic analysis

Inhibition of the purified solubilized mitochondrial activity by *N*-acetylglucosamine (Fig. 2) gave kinetic plots similar to those observed for the activity in intact mitochondria but different from those shown by the purified cytoplasmic activity (Bachelard *et al.*, 1971). Fig. 2(a) shows representative double-reciprocal plots of $1/v$ versus $1/[glucose]$ at varied concentrations of *N*-acetylglucosamine; Fig. 2(c) is the secondary plot of slopes versus [inhibitor]; the K_i^{slopes} value obtained was 0.31 mM. Fig. 2(b) shows a representative plot of $1/v$ versus $1/[MgATP^{2-}]$ at various concentrations of *N*-acetylglucosamine, with secondary plots of slopes and intercepts versus [inhibitor] given as Fig. 2(d). The derived constants were $K_i^{slopes} = 1.4$ mM, $K_i^{intercepts} = 1.1$ mM.

Table 3 gives the mean values for these constants from duplicate experiments where the results were obtained by using Cl^- or acetate as the anion throughout. Values for K_i and K_i' , calculated from the values of K_i^{slopes} and $K_i^{intercepts}$ (see Thompson & Bachelard, 1976) and the substrate kinetic constants, are compared in Table 4 with the equivalent values obtained for the intact mitochondria and for the cytoplasmic enzyme. The analogous values for AMP inhibition of the three enzyme preparations have been calculated from our previous data and are also shown in Table 4.

Discussion

Subsequent analysis of our previous data (Bachelard *et al.*, 1971) suggested that the relationship between substrate constants for the mitochondrial and cytoplasmic activities might be different; this is shown more clearly, and is confirmed, by the results presented here. K_m^{Glc} is the same for the two activities, whereas K_m^{ATP} is not. The differences are more striking

from the analysis of the K_s^{Glc} and K_s^{ATP} values (Table 2): the mitochondrial activity had a K_s^{Glc} of 0.018 mM and K_s^{ATP} of 0.20 mM, in contrast with the higher values obtained with the cytoplasmic activity ($K_s^{Glc} = 0.055$ mM and $K_s^{ATP} = 0.43$ mM).

The results of Table 2 also show that the values for the derived kinetic constants, at least for the solubilized mitochondrial enzyme, are not affected if Cl^- is replaced by acetate.

This possibility of kinetic differences between the mitochondrial and cytoplasmic activities is strongly supported by the results of the dead-end-inhibition kinetic studies. The K_i values for inhibition by both AMP and *N*-acetylglucosamine were similar in the two mitochondrial preparations examined (particulate-bound and solubilized) but different from those exhibited by the cytoplasmic enzyme (Table 4). This difference between the cytoplasmic enzyme and the mitochondrial activities was shown also by the values obtained for K_i' (Table 4). A further major difference observed was in the relationship of K_i to K_i' . The cytoplasmic enzyme gave K_i' values very similar to those for K_i , in contrast with both of the mitochondrial preparations, where K_i was clearly not equal to K_i' (Table 4). The clear difference in these kinetic properties between the cytoplasmic and solubilized mitochondrial activities indicate that the cytoplasmic activity is not an artifact of preparation.

The results show that for the cerebral mitochondrial enzyme, values for K_i^{slopes} and $K_i^{intercepts}$ are clearly different from the values for K_i or K_i' and therefore provide a good practical example of our theoretical treatment of the derivation of true K_i values (Thompson & Bachelard, 1976). In reaction mechanisms of the type shown by cerebral type-I hexokinase (rapid-equilibrium random), apparent K_i values from Dixon plots (Dixon, 1953) and K_i^{slopes} values, can

Fig. 2. *N*-Acetylglucosamine inhibition of solubilized cerebral mitochondrial hexokinase (a) Plot of $1/v$ versus $1/[glucose]$ at a constant concentration of 2mM-MgATP^{2-} and *N*-acetylglucosamine concentrations of: $\circ$, 2.0mM ; $\bullet$, 1.0mM ; Δ , 0.5mM ; $\blacksquare$, 0.2mM ; $\square$, 0mM . (b) Plot of $1/v$ versus $1/[MgATP^{2-}]$ at a constant concentration of 0.1mM-glucose and *N*-acetylglucosamine concentrations of: $\circ$, 2.0mM ; $\bullet$, 1.0mM ; Δ , 0.5mM ; $\blacksquare$, 0.2mM ; $\square$, 0mM . (c) Secondary plot of slopes from (a) versus $[N\text{-acetylglucosamine}]$. (d) Secondary plots of slopes, $\bullet$, and intercepts, $\circ$, from (b) versus $[N\text{-acetylglucosamine}]$. Experimental details are described in the text.

Table 3. Kinetic constants for *N*-acetylglucosamine inhibition of purified solubilized mitochondrial hexokinase. The results are the means from duplicate experiments. Mg^{2+} concentration was 6.75mM (total) with Cl^- as anion, or 5.0mM total with acetate as anion. K_i^{slopes} was obtained as the abscissal intercept from the secondary plot of slopes (of plots of $1/v$ versus $1/[substrate]$) versus the relevant [inhibitor]. Similarly, $K_i^{intercepts}$ was obtained from the secondary plot of intercepts versus [inhibitor].

Total Mg^{2+} (mM)	Anion	Substrate varied	K_i^{slopes} (mM)	$K_i^{intercepts}$ (mM)
6.75	Chloride	Glucose	0.37	—*
		$MgATP^{2-}$	1.66	0.97
5.0	Acetate	Glucose	0.25	—*
		$MgATP^{2-}$	1.51	0.55

* Common intercept on ordinate,

only give the true K_i value if $K_i = K_i'$; this is consistent with the results which we have obtained here. By using the derived values for K_i and K_i' , we calculated the value of the expression:

$$\frac{K_s^A K_m^B + K_m^A [B]}{(K_s^A K_m^B / K_i) + (K_m^A [B] / K_i')}$$

which we showed (Thompson & Bachelard, 1976) to be yielded by the Dixon plot or the slopes re-plot in competitive inhibition of this type of enzyme. This calculation used values derived from two separate sets of experiments (K_s^A , K_m^A , K_m^B from substrate kinetic studies; K_i and K_i' from non-competitive dead-end-inhibition studies). The result was then compared with the observed value objectively derived in a third set of experiments (K_i^{slopes} from the slopes re-plots of competitive dead-end-inhibition studies).

Table 4. *Calculated K_i and K_i' values for inhibition of cerebral cortex hexokinase*

K_i was calculated from $K_i^{\text{slopes}} = K_i[1 + ([\text{Glc}]/K_m^{\text{Glc}})]$ and K_i' from $K_i^{\text{intercepts}} = K_i'[1 + ([\text{Glc}]/K_m^{\text{Glc}})]$ for *N*-acetylglucosamine inhibition. K_i was calculated from $K_i^{\text{slopes}} = K_i[1 + ([\text{ATP}]/K_m^{\text{ATP}})]$ and K_i' from $K_i^{\text{intercepts}} = K_i'[1 + ([\text{ATP}]/K_m^{\text{ATP}})]$ for AMP inhibition (see Thompson & Bachelard, 1976). The anion species was Cl^- ; Mg^{2+} concentration, as MgCl_2 , was 6.75 mM. The results are calculated from the data of Bachelard *et al.* (1971), except for those marked *, which are derived from the extra analyses (means of duplicate experiments) which were performed to complete the comparison.

Inhibitor	Enzyme preparation	K_i (mM)	K_i' (mM)
<i>N</i> -Acetylglucosamine	Cytoplasmic	0.60	0.58
	Intact mitochondria	0.15	0.40
	*Purified solubilized mitochondrial hexokinase	0.21	0.40
AMP	Cytoplasmic	2.4	1.9
	Intact mitochondria	1.4	3.7
	Purified solubilized mitochondrial hexokinase	1.5	3.4

The calculated and observed values agreed overall within 15%, which provided good evidence of internal consistency of our measurements and gave us confidence that the differences in kinetic constants that we have shown are real.

An earlier treatment, in allosteric terms, of the relief of ATP^{4-} inhibition of cerebral cytoplasmic hexokinase by elevated glucose concentrations (Bachelard, 1971) was subsequently suggested to be interpretable in non-allosteric terms if K_i was not equal to K_i' , or K_m^{ATP} not equal to K_s^{ATP} (Purich & Fromm, 1972). The present study shows that $K_m^{\text{ATP}} = K_s^{\text{ATP}}$ and K_i is similar to K_i' in dead-end inhibition of the cytoplasmic activity by both AMP and *N*-acetylglucosamine. An analogous study of ATP^{4-} inhibition has not been performed. If K_i was found to be similar to K_i' in this case, this would also support the allosteric interpretation. The non-identity of cytoplasmic and mitochondrial type-I hexokinase is interesting in relation to speculations that have been advanced on the possibility that changes in the subcellular distribution of cerebral hexokinase may form part of the mechanisms regulating glycolytic rates in the brain. These discussions (Rose & Warms, 1967; Wilson, 1968; Hochman & Sacktor, 1973) were based on the assumption that the mitochondrial and cytoplasmic enzymes are identical, and need re-appraisal if this is not the case. An alternative view is that the mitochondrial hexokinase activity has nothing to do with cerebral glycolysis in itself (Bachelard, 1967), but may fulfil a quite different role. For example, it has been suggested that the mitochondrial hexokinase might act to produce ADP within the mitochondrion as the substrate for oxidative phosphorylation (Moore & Jöbsis, 1970; see also Løvtrup & Svennerholm, 1963; Milstein *et al.*, 1968). A further possibility is a role in adenine nucleotide translocation across the mitochondrial membrane, but as yet there is little evidence for this.

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References

- Bachelard, H. S. (1967) *Biochem. J.* **104**, 286–292
- Bachelard, H. S. (1971) *Biochem. J.* **125**, 249–254
- Bachelard, H. S. & Goldfarb, P. S. G. (1969) *Biochem. J.* **112**, 579–586
- Bachelard, H. S., Clark, A. G. & Thompson, M. F. (1971) *Biochem. J.* **123**, 707–715
- Bigl, V., Müller, L. & Biesold, D. (1971) *J. Neurochem.* **18**, 721–727
- Dixon, M. (1953) *Biochem. J.* **55**, 170–171
- Florini, J. R. & Vestling, C. S. (1957) *Biochim. Biophys. Acta* **25**, 575–578
- Grossbard, L. & Schimke, R. T. (1966) *J. Biol. Chem.* **241**, 3546–3560
- Hochman, M. S. & Sacktor, B. (1973) *Biochem. Biophys. Res. Commun.* **54**, 1546–1553
- Johnson, M. K. (1960) *Biochem. J.* **77**, 610–618
- Kellog, E. W., Knoll, H. R. & Wilson, J. E. (1974) *J. Neurochem.* **22**, 461–463
- Løvtrup, S. & Svennerholm, L. (1963) *Exp. Cell Res.* **29**, 298–313
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Milstein, J. M., White, J. G. & Swaiman, K. F. (1968) *J. Neurochem.* **15**, 411–415
- Moore, C. L. & Jöbsis, F. F. (1970) *Arch. Biochem. Biophys.* **138**, 295–305
- Purich, D. L. & Fromm, H. J. (1972) *Biochem. J.* **130**, 63–69
- Redkar, V. D. & Kenkare, U. W. (1972) *J. Biol. Chem.* **247**, 7576–7584
- Rose, I. A. & Warms, J. V. B. (1967) *J. Biol. Chem.* **242**, 1635–1645
- Sottocasa, G. L., Kuylenssterna, B., Ernster, L. & Bergstrand, A. (1967) *J. Cell Biol.* **32**, 415–438
- Stern, J. (1954) *Biochem. J.* **58**, 536–542
- Thompson, M. F. & Bachelard, H. S. (1970) *Biochem. J.* **118**, 25–34
- Thompson, M. F. & Bachelard, H. S. (1976) *Anal. Biochem.* **71**, 507–518
- Vallejo, C. G., Marco, R. & Sebastian, J. (1970) *Eur. J. Biochem.* **14**, 478–485
- Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324–332
- Wilson, J. E. (1968) *J. Biol. Chem.* **243**, 3640–3647