Mitochondrial Membrane-Bound Hexokinase of Ascites Tumor Cells

Functional Implications of Lysine Residues Studied by Modification with Imidoesters

Pedro A. LAZO and Lisardo BOSCA

Instituto de Enzimología y Patología Molecular del C.S.I.C. Facultad de Medicina. Universidad Autónoma de Madrid

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Summary: Conformational changes of hexokinase from ascites tumor cells have been studied by chemical modification of lysine residues with imidoesters with the following results:

- 1) The membrane-bound enzyme, in contrast to the soluble enzyme, is not inactivated by treatment with dimethyl suberimide, which suggests (a) lysine residue(s) essential for the activity that is protected in the membrane-bound enzyme.
- 2) Three different conformations have been detected in the membrane-bound enzyme. Two

of these are induced by glucose and glucose 6-phosphate, respectively.

- 3) Treatment of the membrane-bound enzyme with dimethyl suberimide affects its sensitivity to the inhibition by glucose 6-phosphate, but not its activity or degree of maximal inhibition. This suggests that lysine(s) is related to the binding of glucose 6-phosphate to its allosteric regulatory site.
- 4) In intact tumor cells, most, if not all, of the hexokinase activity seems to be in a membrane-bound form.

Mitochondriale, membrangebundene Hexokinase aus Ascites-Tumorzellen. Untersuchungen zur funktionellen Bedeutung von Lysin-Resten durch Modifikation mit Imidoestern

Zusammenfassung: Konformationsänderungen der Hexokinase aus Ascites-Tumorzellen wurde mit Hilfe chemischer Modifikationen der Lysin-Reste durch Imidoester untersucht. Die Ergebnisse waren folgende:

- 1) Das membrangebundene Enzym wird im Gegensatz zum löslichen Enzym nicht durch Behandlung mit Dimethylsuberimidat inaktiviert. Das

deutet auf (einen) Lysin-Rest(e) hin, die essentiell sind für die Aktivität und beim membrangebunden Enzym geschützt vorliegen.

- 2) Für das membrangebundene Enzym wurden drei verschiedene Konformationen gefunden. Zwei von ihnen werden durch Glucose bzw. Glucose-6-phosphat induziert.

Enzymes:

Hexokinase, ATP:D-hexose 6-phosphotransferase (EC 2.7.1.1);
Lactate dehydrogenase, lactate:NAD⁺ oxidoreductase (EC 1.1.1.27);
Pyruvate kinase, ATP:pyruvate 2-O-phosphotransferase (EC 2.7.1.40).

Abbreviations:

Sbi(OMe)₂ = Dimethyl suberimide dihydrochloride;

Tes = 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulphonic acid; for Reagents 2–5 see "Materials".

3) Behandlung des membrangebundenen Enzyms mit Dimethylsuberimide beeinflusst seine Empfindlichkeit gegenüber einer Hemmung mit Glucose-6-phosphat, aber nicht seine Aktivität und nicht seine maximale Hemmbarkeit. Das läßt vermuten, daß eine Beziehung besteht zwischen

(einem) Lysin-Rest(en) und der Bindung von Glucose-6-phosphat an dessen allosterischen Regulationsort.

4) In intakten Tumorzellen scheint die meiste, wenn nicht die gesamte Hexokinase-Aktivität in membrangebundener Form vorzuliegen.

Key words: Hexokinase; lysine residues; conformational changes; ambiquitous enzymes; tumor.

It is well established that proteins, when binding a ligand, are able to undergo a conformational change. Such changes have been identified in yeast hexokinase by functional^[1] and structural studies^[2]. In mammals, the most studied hexokinase is isoenzyme I from brain, reviewed by Wilson^[3], which exists in a soluble and particulate form^[4,5]. The enzymes that can be found in these two forms have been called ambiquitous enzymes^[3]. Conformational changes in a soluble purified preparation of the brain isoenzyme have been determined by the study of kinetic parameters after several kinds of treatment: using proteolytic enzymes^[6,7], physical agents^[6,7], and group-specific reagents^[6-8]. As a result, a functional model of the enzyme has been proposed^[3,6].

In this report we have studied the possibility of the existence of such conformational changes in the isoenzyme II of animal hexokinase from ascites tumor cells, which also exists in a soluble and mitochondrial membrane-bound form^[9]. The change from one form to the other can have an effect on its kinetic properties^[10]. This isoenzyme is consequently an ambiquitous enzyme, though its physiological significance has not yet been established. Almost nothing is known about its primary structure, subunit composition and three-dimensional structure. Only recently some studies have been performed regarding the nature of its active site^[11,12]. We have treated this isoenzyme with different kinds of monofunctional and bifunctional imidoesters^[13] in the presence of different ligands, in order to catch different conformations of the enzyme in a stable form. Kinetic parameters were used as markers of such possible conformational changes. For these studies we have used mitochondrial membranes, since no in vitro reconstitution system

for the particulate enzyme has yet been developed. The results reported here have improved our understanding of the role played by different lysine groups in different aspects of enzyme function: binding to mitochondrial membrane, conformational changes and catalytic and regulatory processes. Nevertheless the specific identification of the residues involved would require studies with a purified enzyme using radiolabelled group-specific reagents followed by sequence analysis.

The cross-linking technique has been applied to intact ascites tumor cells to detect the intracellular location of the enzyme.

Materials and Methods

Materials

Cross-linking reagents:

- 1) Dimethyl suberimide dihydrochloride [= Sbi-(OMe)₂];
- 2) dimethyl 3,3'-dithiobis(propionimide) dihydrochloride;
- 3) dimethyl *N,N'*-bis(2-(carboximido)ethyl)tartrimidate dihydrochloride;
- 4) sodium 2-(1-iminoethoxy)ethanesulphonate hydrochloride and
- 5) 3,3'-dithiobis[*N*-(propionyloxy)succinimide].

All five reagents were from Pierce, Rockford, IL, U.S.A. Digitonin and Tes buffer (2-[12-hydroxy-1,1-bis(hydroxymethyl)ethyl] amino)ethanesulphonic acid) were from Calbiochem, San Diego, Ca., U.S.A. Glucose and sucrose were from May & Baker, Dagenham, Essex, U.K. Triethanolamine and EDTA were from Merck, Darmstadt, West Germany. All other reagents were from Sigma, St. Louis, Mo., U.S.A.

Cells and enzyme preparations

A hyperdiploid strain of Ehrlich-Létré ascites carcinoma (kindly supplied by Dr. J. Coll, Centro Especial

Ramón y Cajal, Madrid) was grown in the abdominal cavity of 2-month old male Swiss mice, by weekly inoculation of 0.1 ml of ascitic fluid. Cells were collected and mitochondria prepared as described by Lazo and Sols^[14]. The mitochondrial fraction in these conditions contained 80% of the total hexokinase activity. The supernatant was centrifuged at 40000 $\times$ g for 30 min in a Sorvall RC-2B with an SS-34 rotor, and the hexokinase in this cytosolic supernatant was considered as the soluble form of the enzyme.

The membrane-bound preparation behaved as a single population of hexokinase II, characterized by the kinetic parameters for the substrates and the lack of any effect of P_i on the inhibition by glucose 6-phosphate^[15]. This observation is confirmed by a different group^[16].

Treatment of particulate and soluble hexokinase

Mitochondria containing 7–10 mg of protein were suspended in 0.2 ml of 0.1M triethanolamine, 75mM NaCl, pH 8.5. To this suspension, 1.8 ml of the same buffer containing 6 mg of imidoester, was added. The solution of imidoester was prepared, taking great care to prevent the pH from falling below pH 8.5; for this 2M NaOH was used. The final mitochondrial suspension was incubated at 30 °C for 30 min in the presence or absence of ligands, as indicated in the Results and Discussion section. The reaction was stopped by adding 40 ml of cold 0.25M sucrose, 2mM EDTA, 2mM 2-mercaptoethanol, 10mM triethanolamine, pH 7.4, and the suspension was centrifuged at 10000 $\times$ g for 15 min to collect the mitochondria which were resuspended in this buffer to a final concentration of 20% (w/v).

This procedure was common for all imidoesters used. The drop in pH by the addition of buffer inactivates the imidoesters. These mitochondrial suspensions were used for enzymatic assays. When Reagent 3 was used, a parallel experiment was done in which, after treatment of the enzyme, the alcohol groups were oxidized by treatment with 3mM sodium periodate during 5 min at 30 °C in the above mentioned buffer, to break the cross-link. The reaction was stopped by the addition of 100 ml of cold buffer and centrifugation as above, otherwise, periodate could lead to the inactivation of hexokinase in approx. 45 min. Similarly, samples which have been modified by bifunctional reagents containing disulphide bridges (Reagent 2 or 5) were subsequently treated with 5mM dithioerythritol to cleave the cross-link^[17].

The soluble enzyme was treated as follows: 0.2 ml of the cytosolic supernatant plus 1.8 ml of $Sb(OMe)_2$ solution was incubated under identical conditions to the mitochondrial fraction, but the reaction was stopped by carefully lowering the pH to 7.4 with 0.1M HCl. This solution was used for enzymatic determinations.

Cross-linking of cells

Ascites tumor cells were cross-linked with $Sb(OMe)_2$ following the general procedure of Aragón et al.^[18], except that 30 mg of $Sb(OMe)_2$ per g of cells and 0.2% digitonin was used.

Enzyme assays

Hexokinase was spectrophotometrically assayed using 1 U of pyruvate kinase and 1 U lactate dehydrogenase, 3mM phosphoenolpyruvate, 0.2mM NADH, 5mM glucose and 0.5mM Mg ATP in a mixture containing 0.1M KCl, 5mM $MgCl_2$, 5mM 2-mercaptoethanol, 50mM Tes buffer, pH 7.4 in 1 ml of final volume, at 37 °C. The activity was recorded in a 2400 Gilford spectrophotometer. Lactate dehydrogenase and pyruvate kinase were assayed as described by Aragón et al.^[18]. Protein was determined by the dye-binding method of Bradford^[19].

Results and Discussion

Treatment with dimethyl suberimide

The cross-linking results with $Sb(OMe)_2$ are summarized in the table. First of all it is important to note that the soluble enzyme is completely inactivated, while the membrane-bound form retains its activity. This suggests that there is (a) lysine group(s) not accessible to $Sb(OMe)_2$ in the membrane-bound enzyme, that is required for the catalytic process but is not in the active site, since this site is still accessible in the membrane-bound enzyme. Probably the lysine residue(s) is required for the conformational changes involved in the catalytic mechanism.

Whether this protection is due to its embedment within the lipid environment, or is related to the recently described hexokinase-binding protein in liver mitochondrial^[20] has yet to be established. Ascites tumor hexokinase binds equally well to mitochondrial membrane of different sources^[9]. The membrane-bound form retains its activity when treated with $Sb(OMe)_2$ in the presence or absence of glucose. However, modification in the presence of glucose 6-phosphate fully inactivates the enzyme, suggesting that (a) lysine residue(s) required for the catalytic process becomes accessible to $Sb(OMe)_2$. $Sb(OMe)_2$ does not solubilize the enzyme, and the cross-linked mitochondrial bound hexokinase could not be solubilized by later addition of glucose 6-phosphate.

To test whether hexokinase was being solubilized by glucose 6-phosphate^[5,9] before the imido-

Table. Effect of treatment with imidoesters on the activity and inhibition by glucose 6-phosphate of hexokinase-II. The treatment conditions are described in the Experimental section. For the soluble enzyme, the activity in this fraction before treatment was taken as the reference value. For the intact cross-linked cells, the hexokinase activity in a homogenate is the reference value. Results are means $\pm$ S.E.M. for $n = 9$ in the case of $\text{Sbi}(\text{OMe})_2$ and $n = 6$ with the other reagents. n.d. = not determined.

	Activity	Apparent K_i for glucose 6-phosphate	Activity		
	[%]	[mM]	[%]		
1) Mitochondria control	100 $\pm$ 2	0.09 $\pm$ 0.01			
2) Mitochondria treated with imidoesters	$\text{Sbi}(\text{OMe})_2$		Reagent 4	Reagent 3	Reagent 3 cleaved with 3mM periodate
Imidoester					
Additions					
No additions	91 $\pm$ 2	0.50 $\pm$ 0.03*	35 $\pm$ 3*	96 $\pm$ 3	60 $\pm$ 4*
10mM glucose	90 $\pm$ 3	2.00 $\pm$ 0.10*	91 $\pm$ 2	63 $\pm$ 2*	120 $\pm$ 4*
1mM glucose 6-phosphate	< 5*	—	17 $\pm$ 3*	52 $\pm$ 2*	60 $\pm$ 3*
10mM <i>N</i> -acetylglucosamine + 1mM glucose 6-phosphate	40 $\pm$ 2*	7 $\pm$ 0.20*	15 $\pm$ 3*	52 $\pm$ 3*	63 $\pm$ 4*
3) Soluble enzyme	< 5*	n.d.	n.d.	n.d.	n.d.
4) Intact cross-linked cells	100 $\pm$ 5	0.50 $\pm$ 0.04*	n.d.	n.d.	n.d.

* $P < 0.001$

esters react, a control experiment was performed to see the effect of glucose 6-phosphate under the experimental conditions for the imidoester reaction. The amount of hexokinase in the supernatant, after the 10000 $\times$ g spin, was less than 10% and it could be sedimented at 40000 $\times$ g, suggesting that it is a particulate enzyme derived from broken mitochondria under the harsh conditions for imidoester treatment. A similar effect was observed in the presence of reaction buffer alone. This is what we expected if hexokinase II behaves in a similar way to hexokinase I under equivalent conditions of ionic strength and temperature^[21]. The alternative possibility, that soluble, inactivated hexokinase could displace the equilibrium can be ruled out by the above information, as well as from the data with other reagents (see table).

Therefore the inactivation is happening in the membrane-bound form, but whether this lysine residue(s) is the same as that exposed in the

soluble enzyme, cannot be determined in these experiments. *N*-Acetyl-D-glucosamine, which binds to the active site but does not induce a conformational change, partly prevents the inactivation achieved in the presence of glucose 6-phosphate.

When the membrane-bound enzyme was modified in the presence or absence of glucose, the sensitivity of the enzyme to the competitive (vs. ATP) inhibition by glucose 6-phosphate is decreased, particularly when treated in the presence of glucose. This suggests that there is a lysine group close to the allosteric site for glucose 6-phosphate^[22,23] involved in the affinity for the effector, but not in the inhibitory mechanism since full inhibition can still be achieved. The apparent K_m for glucose and MgATP were not affected in the modified enzyme.

All these data suggest the existence of at least three different possible conformations in mem-

brane-bound hexokinase II: one in the absence of ligands, and the other two when complexed with glucose or glucose 6-phosphate, respectively. This is in agreement with the results from the brain isoenzyme, where these ligands can induce different conformational changes^[3,6-8].

Treatment with sodium 2-(1-iminoethoxy)-ethanesulphonate hydrochloride

To test whether the effects described above were a consequence of blocking lysine amino groups or of cross-linking, the enzyme was treated with the monofunctional imidoester 2-(1-iminoethoxy)-ethanesulphonate (= Reagent 4, see table). Glucose protects the enzyme from inactivation by this reagent. Even in the absence of any addition, Reagent 4 drops the activity much more than does treatment with $\text{Sbi}(\text{OMe})_2$, suggesting that the free and glucose-complexed forms of the enzyme are different. In general the results observed with bifunctional reagents, are a consequence of both the blocking of amino groups and of the strain imposed by the cross-linking. An alternative explanation might be that $\text{Sbi}(\text{OMe})_2$ reacts monofunctionally and the differences are due to the different size of the side chains of the reagents.

Treatment with other bifunctional reagents

Treatment with dimethyl *N,N*-bis[2-(carboximido)-ethyl]tartarimidate dihydrochloride (Reagent 3, see table), a cleavable bis-imidoester^[28], seems to have no effect on enzyme activity when no ligand is present. However, the addition of glucose or glucose 6-phosphate decreases the enzyme activity by a factor of 2. After cross-linking, the imidoester was cleaved with 3mM periodate, converting it to monofunctional and liberating the enzyme of the strain imposed by the cross-link. The results obtained were similar except in free and glucose-complexed enzyme. From the data in both situations we can conclude that the conformations of free, glucose-complexed and glucose 6-phosphate-complexed enzyme are different.

Other bifunctional reagents were tested: dimethyl 3,3'-dithiobis(propionimide) and 3,3'-dithiobis[*N*-(propionyloxy)succinimide]. Both contain a cleavable disulphide bridge between the two

reactive groups. Both lead to full inactivation of the enzyme irrespective of the ligands present. After modification of the reagents with 5mM dithioerythritol (which results in reduction of the disulphide bridge) no reactivation was detected.

Localization of hexokinase in intact cross-linked cells

We used the fact that there is a very marked difference in lysine reactivity between the soluble and membrane-bound hexokinase to investigate the localization of the enzyme in cross-linked cells. When intact cells were treated with $\text{Sbi}(\text{OMe})_2$, the results were similar (see table), (concerning activity and sensitivity to glucose 6-phosphate) to that of membrane-bound hexokinase not bound to any ligand. The possibility that this difference was due to a permeability problem of $\text{Sbi}(\text{OMe})_2$ was ruled out by measuring the activity of other cytosolic enzymes in the treated cells. Lactate dehydrogenase and pyruvate kinase were both 80% inactivated. These activities were localized inside the cross-linked cells, since after centrifugation the activities were associated with the pellet, ruling out the possibility of leaking cells. These results suggest that most, if not all, of hexokinase II in ascites tumor cells exists in a membrane-bound form.

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Literature

- 1 De La Fuente, G., Lagunas, R. & Sols, A. (1970) *Eur. J. Biochem.* **16**, 226-233.
- 2 Bennet, W. S. & Steitz, T. A. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4848-4852.
- 3 Wilson, J. E. (1980) *Curr. Top. Cell. Regul.* **16**, 1-54.
- 4 Crane, R. K. & Sols, A. (1953) *J. Biol. Chem.* **203**, 273-292.
- 5 Wilson, J. E. (1968) *J. Biol. Chem.* **243**, 3640-3647.
- 6 Wilson, J. E. (1978) *Arch. Biochem. Biophys.* **185**, 88-99.
- 7 Wilson, J. E. (1979) *Arch. Biochem. Biophys.* **196**, 79-87.
- 8 Chou, A. C. & Wilson, J. E. (1974) *Arch. Biochem. Biophys.* **163**, 191-199.

- 9 Rose, I. A. & Warms, J. V. B. (1967) *J. Biol. Chem.* **242**, 1635–1645.
- 10 Kosow, D. P. & Rose, I. A. (1968) *J. Biol. Chem.* **243**, 3623–3630.
- 11 Lazo, P. A. & Sols, A. (1979) *FEBS Lett.* **98**, 88–90.
- 12 Connolly, B. A. & Trayer, I. P. (1979) *Eur. J. Biochem.* **93**, 375–385.
- 13 Peters, K. & Richards, F. M. (1977) *Annu. Rev. Biochem.* **46**, 523–551.
- 14 Lazo, P. A. & Sols, A. (1980) *Biochem. J.* **190**, 705–710.
- 15 Colowick, S. P. (1973) *The Enzymes* (Boyer, P. D., ed.) vol. 9, pp. 1–48, Academic Press, New York.
- 16 Rose, I. A. & Warms, J. V. B. (1982) *Arch. Biochem. Biophys.* **213**, 625–634.
- 17 Lomant, A. J. & Fairbanks, G. (1976) *J. Mol. Biol.* **104**, 243–261.
- 18 Aragon, J. J., Feliu, J. E., Frenkel, R. N. & Sols, A. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6324–6328.
- 19 Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254.
- 20 Felgner, P. L., Messer, J. L. & Wilson, J. E. (1979) *J. Biol. Chem.* **254**, 4946–4949.
- 21 Felgner, P. L. & Wilson, J. E. (1977) *Arch. Biochem. Biophys.* **182**, 282–294.
- 22 Crane, R. K. & Sols, A. (1954) *J. Biol. Chem.* **210**, 597–606.
- 23 Lazo, P. A., Sols, A. & Wilson, J. E. (1980) *J. Biol. Chem.* **255**, 7548–7551.
- 24 Coggins, J. R., Hooper, E. A. & Perham, R. N. (1976) *Biochemistry* **15**, 2527–2533.

Dr. Pedro A. Lazo, Instituto de Enzimología y Patología Molecular del C.S.I.C., Facultad de Medicina, Universidad Autónoma de Madrid, Arzobispo Morcillo 4, Madrid 34, Spain.

Present address: Department of Biochemistry University of Cambridge, Tennis Court Road, Cambridge, CB2 1QW, U.K.