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# Production of a specific antibody against pyruvate kinase type M2 using a synthetic peptide

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The pyruvate kinase isozymes M1 and M2 are structurally and immunologically closely related. To obtain an antibody which discriminates between these two forms, a synthetic tetradecapeptide with a sequence specific for pyruvate kinase type M2 from rats was constructed. Antisera from rabbits, immunized with this peptide, reacted specifically with the M2-type holoenzyme of both rat and human origin, and did not cross-react with the M1-type isozyme. This was established by immunoblot analysis, both under dissociating and non-dissociating conditions.

Pyruvate kinase; Isozyme; Immunoblotting; Peptide antibody

## 1. INTRODUCTION

Pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) catalyzes one of the last steps in glycolysis, the conversion of phospho*enol*pyruvate to pyruvate. Four isozymes have been described in mammalian tissues, named the M1, M2 (or K), L, and R type [1,2].

Pyruvate kinase (PK) is a tetramer, and when two or more subunit types are synthesized simultaneously within the same cell, hybrid isozymes are formed [3]. These hybrids, of which the subunit composition is designated by subscripts e.g. $(M2)_3M1$, can easily be separated electrophoretically.

The M1 and M2 isozymes are structurally and immunologically closely related [4] and it has been shown that these two forms are produced by two different mRNAs, transcribed from the same gene [5,6]. Recently, Noguchi et al. [7,8] determined the complete nucleotide sequences for both M1- and M2-type PK from rat by sequencing the cDNAs. The derived amino acid sequences turned out to be identical except for one region of 45 residues. Even within this region the M1 and M2 sequences show a high degree of homology and therefore it is not surprising that conventional antibodies raised against the M2 type cross-react with the M1 type, and vice versa.

In order to obtain antibodies specific for the M2 isozyme, we constructed a synthetic tetradecapeptide. The sequence of this peptide was chosen from the M2-type specific region of rat PK, as elucidated by Noguchi et al. [8]. Immunization of rabbits with this peptide, coupled to keyhole limpet hemocyanin as a carrier protein, resulted in antibodies not only reacting with the peptide, but also with the M2-type isozyme and not cross-reacting with the M1 isozyme.

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*Abbreviations:* PK, pyruvate kinase; AMTC, anaplastic medullary thyroid carcinoma (rat); DMTC, differentiated medullary thyroid carcinoma (rat); KLH, keyhole limpet hemocyanin; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; EDAC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride

## 2. MATERIALS AND METHODS

Substrates, nucleotides and auxiliary enzymes for the determination of pyruvate kinase activity and fructose 1,6-bisphos-

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phate were obtained from Boehringer (Mannheim, FRG). Dithiothreitol and keyhole limpet hemocyanin (KLH) were from Sigma (St. Louis, MO). SDS, acrylamide, bisacrylamide and EDAC were from Bio-Rad (Richmond, CA). All other chemicals used were of the highest purity available.

2.1. *Production of antipeptide antibodies*

A tetradecapeptide was synthesized by standard solid-phase methods. The sequence of this peptide is shown in fig.1.

The crude preparation was loaded on a Sep-Pak C18 cartridge (Waters Associates, Milford, MA) in 0.1% trifluoroacetic acid; the peptide was eluted with 0.1% trifluoroacetic acid in 50% acetonitrile and lyophilized.

The synthesized peptide was coupled to KLH as a carrier protein, using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl as the coupling reagent. KLH (5.4 mg in 0.54 ml $H_2O$) and peptide (5 mg in 0.5 ml $H_2O$) were mixed and EDAC (11 mg in 0.1 ml phosphate buffered saline) was added. Coupling was allowed to proceed for 2 h at room temperature under rotation. Consecutively, the mixture was extensively dialyzed against saline to remove excess of EDAC. Antisera against the peptide were prepared by priming two New Zealand white rabbits via intramuscular injections with 1 mg of the peptide-carrier conjugate in 1 ml saline, emulsified with 1 ml complete Freund's adjuvant (Gibco, Grand Island, NY). After three weeks, the rabbits were boosted with 1 mg of the immunogen emulsified in Freund's incomplete adjuvant and boosted again at 1 month intervals.

The titer against the peptide was measured by ELISA, using peptide or peptide conjugated to bovine serum albumin as the antigen [9,10].

Twelve weeks after the first immunization the sera of both rabbits reacted specifically with the peptide. The serum titers could not be increased by a further booster and therefore both rabbits were bled at week thirteen and the sera were collected.

2.2. *Preparation of tissue and cell extracts*

Differentiated and anaplastic medullary thyroid carcinomas from rats (DMTC and AMTC, respectively) were maintained as described elsewhere [11]. The human glioma cell line HS 683 and the rat glioblastoma cell line C-6 (American Type Culture Collection, Rockville, MD) were cultured as described in [12].

Tumorous and normal tissues were homogenized in 3 vols of ice-cold extraction buffer containing 50 mM Tris-HCl (ph 7.5), 0.1 M KCl, 0.5 M sucrose, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM diisopropylfluorophosphate and 1 mM fructose 1,6-bisphosphate. Cell pellets ($2 \times 10^7$ cells) were homogenized by sonication in 0.3 ml of the same buffer. The homogenates were centrifuged (20 min, $40000 \times g$) and the supernatants (soluble fraction) and pellets (particulate fraction) were stored at $-80°C$.

2.3. *Purification of pyruvate kinase isozymes*

PK type M2 was purified from AMTC by chromatography on a Blue-Sepharose CL-6B column (Pharmacia, Uppsala, Sweden) [13]. PK type R was partially purified from the hemolysate of human erythrocytes by a fractionated ammonium sulfate precipitation [14].

PK activity was measured as described by Bücher and Pfleiderer [15].

2.4. *Electrophoresis and immunoblotting*

SDS-electrophoresis was carried out in 11% polyacrylamide gels according to Laemmli [16]. Gels were either stained with Coomassie brilliant blue or used for immunoblotting.

Separated proteins were electrophoretically transferred (2 h, 60 V, 4°C) to nitrocellulose (Schleicher and Schüll, Kassel, FRG) in 25 mM Tris/192 mM glycine (pH 8.3) and 20% methanol (v/v) using a Bio-Rad Trans-Blot apparatus. After blocking the remaining binding sites with 10 mM Tris-HCl (pH 7.4), 0.9% NaCl, 1% gelatin and 0.05% Tween-20, the blots were incubated (2 h, 20°C) with the antipeptide antiserum, diluted 1:2000 in the same buffer. The sheets were then treated with peroxidase conjugated goat anti-rabbit IgG (Nordic, Tilburg, The Netherlands) (1 h, 20°C), washed and the peroxidase visualized by chloronaphthol staining.

Separation of PK isozymes by electrophoresis on cellulose acetate strips and subsequent staining was performed as described previously [11]. For immunoblot analysis the separated proteins were transferred to nitrocellulose by vacuum blotting in a slabdrier (Pharmacia, Uppsala, Sweden) for 2 h at room temperature. The blot was then treated as described above, except that staining was performed with 3,3′-diaminobenzidine.

## 3. RESULTS

The antiserum, raised against the synthetic peptide with a sequence specific for the M2 subunit of PK from rat tissues, also reacted with the complete, denatured M2 subunit. This was established by subjecting purified PK isozymes as well as tissue or cell extracts from human and rat origin to SDS-PAGE, followed by transfer of the separated proteins to nitrocellulose and immunoblotting (fig.2). Purified M2-type PK from AMTC of rat was clearly recognized by the antiserum (lane 3). In the soluble fraction of the rat glioblastoma cell line C-6 (containing predominantly $(M2)_4$ and a minor amount of $(M2)_3M1$ isozymes, see below) only one protein band with a molecular mass of about 60 kDa was detected by the antipeptide antibodies (fig.2, lane 2), and no protein band at all in the particulate fraction (lane 1). Rat skeletal muscle, which contains the $(M1)_4$ isozyme, did not show any reaction in the immunoblot (lane 4). So, the antibody appeared to be very specific for PK as shown by SDS-PAGE of whole tissue homogenates. Furthermore, the antiserum could specifically recognize the M2 subunit of PK from rats and did not cross-react with the M1 subunit.

Identical results were obtained when using PK from human tissues. The antiserum only reacted with the M2 subunit of PK (from glioma cell line), but not with the M1 (from skeletal muscle) nor with

**M2-type** 388 Ile-Tyr-His-Leu-Gln-Leu-Phe-Glu-Glu-Leu-Arg-Arg-Leu-Ala-Pro-

**M1-type** 388 Val-Phe-His-Arg-Leu-Leu-Phe-Glu-Glu-Leu-Ala-Arg-Ala-Ser-Ser-

Ile-Thr-Ser-Asp-Pro-Thr-Glu-Ala-Ala-Ala-Val-Gly-Ala-Val-Glu-

Gln-Ser-Thr-Asp-Pro-Leu-Glu-Ala-Met-Ala-Met-Gly-Ser-Val-Glu-

Ala-Ser-Phe-Lys-Cys-Cys-Ser-Gly-Ala-Ile-Ile-Val-Leu-Thr-Lys

Ala-Ser-Tyr-Lys-Cys-Leu-Ala-Ala-Ala-Leu-Ile-Val-Leu-Thr-Glu

Fig.1. Amino acid sequences specific to the M1- and M2-type PK, according to Noguchi et al. [8]. The sequence of the constructed synthetic peptide is underlined.

the R subunit (partially purified from erythrocytes) (not shown).

Electrophoresis of PK on cellulose acetate was performed under non-dissociating conditions in order to separate the tetrameric isozymes. DMTC of rat contained both the $(M1)_4$ and the $(M2)_4$ homotetramer as well as the intermediate hybrids (fig.3, lane 1), as can be visualized by staining for enzyme activity. As mentioned before, rat skeletal muscle contained the $(M1)_4$ isozyme (lane 2) and the C-6 cell line the $(M2)_4$ enzyme and some of the $(M2)_3M1$ hybrid (lane 3). The corresponding immunoblot shows that the $(M2)_4$ isozyme from DMTC and C-6 was recognized by the antipeptide antiserum, but not the $(M1)_4$ enzyme from muscle and DMTC (fig.3). The $(M2)_3M1$ hybrid also gave a weak, but visible reaction with the antiserum.

Interestingly, the antiserum revealed a certain

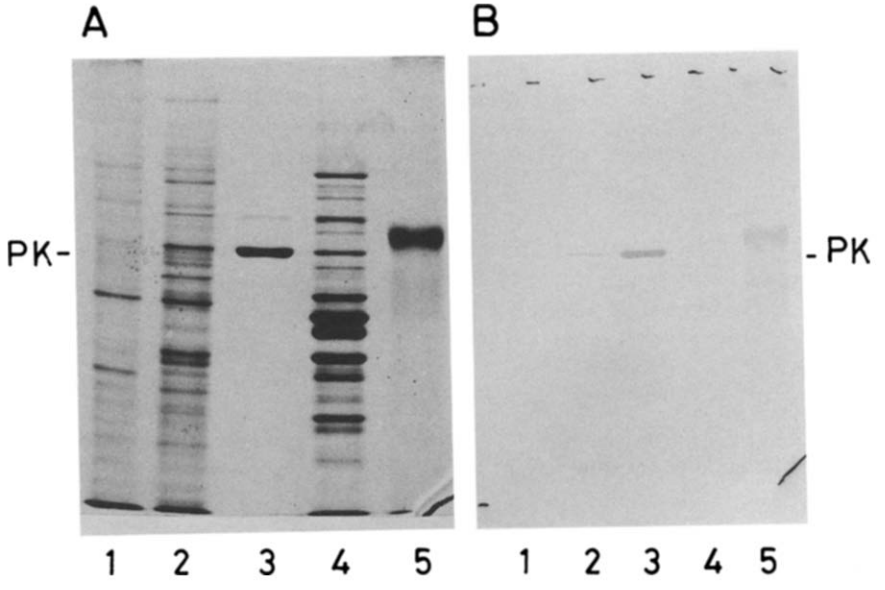


Fig.2. The particulate (lane 1) and the soluble fraction (lane 2) of an extract of C-6 glioblastoma cell line, purified PK type M2 from AMTC (lane 3) and a cytosolic extract of rat skeletal muscle (lane 4), prepared as described in section 2, were resolved by SDS-gel electrophoresis in 11% polyacrylamide. The proteins were either stained with Coomassie blue (A) or transferred to nitrocellulose for reaction with the antipeptide antiserum (B). Samples in lanes 2 and 4 contained equal amounts of PK activity (0.1 U); the sample in lane 3 contained 0.2 U PK activity. Prestained chicken PK was used as standard (lane 5).

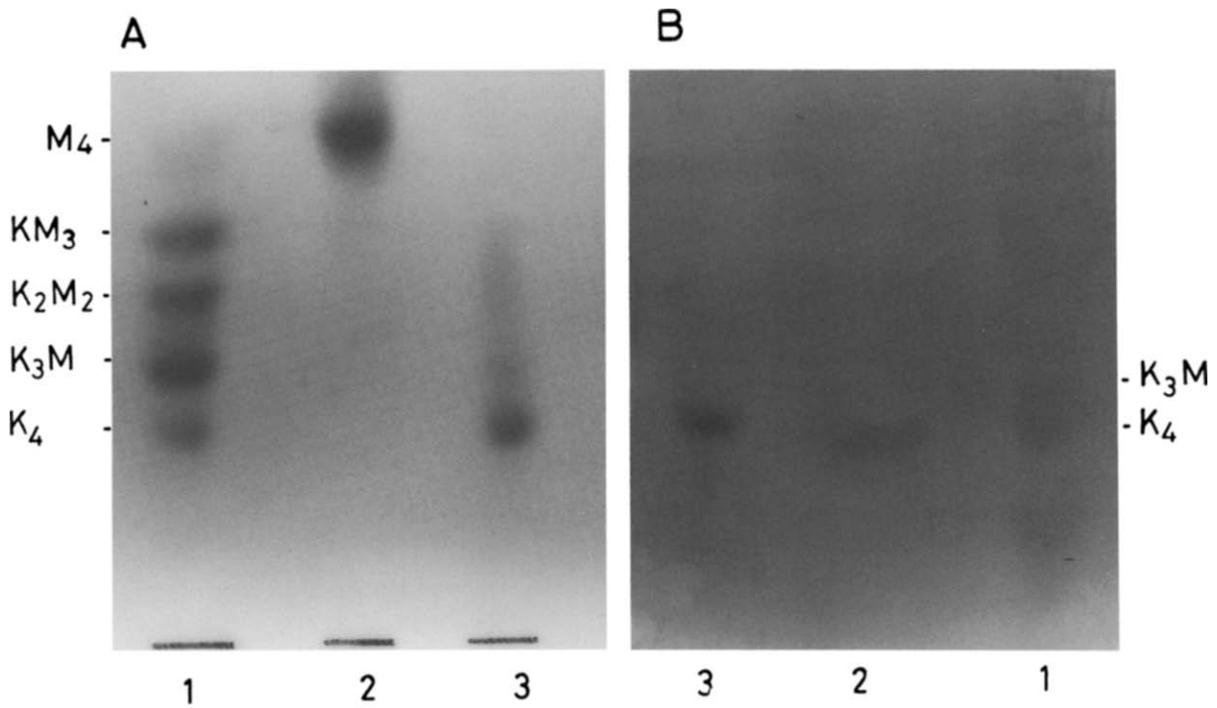


Fig.3. Cytosolic extracts of DMTC (lane 1), rat skeletal muscle (lane 2) and C-6 glioblastoma cell line (lane 3), prepared as described in section 2, were subjected to electrophoresis on cellulose acetate, and the separated isozymes were either visualized by staining for enzyme activity (A) or subjected to Western blotting and subsequent incubated with the antipeptide antiserum (B). Samples contained 10 mU PK activity for activity staining and 30 mU PK activity for blotting. M1 and M2 subunits are designated as M and K, respectively, according to the nomenclature used by Ibsen [2].

amount of $(M2)_4$ in the muscle extract, which was not seen after enzyme activity staining (fig.3, lane 2). This can be explained by the presence of fibroblasts and blood vessels in skeletal muscle tissue, which are known to contain the $(M2)_4$ enzyme. This observation demonstrates the specificity and sensitivity of the antipeptide antiserum for the M2-type enzyme. Again, the same results were obtained on human tissues.

## 4. DISCUSSION

Conventional prepared antibodies raised against M2-type PK cross-react with the M1 type, and vice versa, due to the high homology between the M1 and the M2 isozyme. The elucidation of the complete nucleotide sequences of both M1- and M2-type PK from rat by Noguchi and co-workers [8] prompted us to find a new approach for obtaining specific antibodies against M2-type pyruvate kinase. The primary structures of the M1 and M2 type were identical except for one region of 45 amino acids [8]. From this region a sequence of 14 residues was chosen, in which the differences between the two isozymes were most frequent, and a corresponding synthetic tetradecapeptide with a M2-specific sequence was constructed (see fig.1).

Antibodies obtained after immunization not only reacted with the peptide, but also with the M2-type holoenzyme from rat as well as human origin, both in native tetrameric form and when dissociated in subunits. The antiserum proved to be very specific, as no reaction was found against the M1, L, or R subunit of PK, nor against any other protein. This was demonstrated by immunoblotting experiments after SDS-PAGE and cellulose acetate electrophoresis. These results confirm the M2-type specificity of the synthesized peptide.

It has been suggested by Noguchi et al. [8] that the subunit specific sequence of 45 amino acids of the M2-type PK might be important for mediating the allosteric properties of this isozyme. Therefore,

it might be possible that the antipeptide antiserum, directed against a part of this sequence, had an effect on some kinetic features of the M2 enzyme. However, no alterations in the kinetic behavior of the $(M2)_4$ enzyme could be observed in the presence of the antiserum (not shown).

The applicability of the antibody in immunohistochemistry is now being studied. The antibody may be helpful in understanding the isozymic shift from M1- towards M2-type PK in various tumors [12,17–19], for instance in human gliomas, in which a strong correlation exists between isozyme composition, degree of histological differentiation and postoperative survival [19]. In these studies, the isozyme composition is determined in homogenates of whole tissue specimens, using either electrophoretical methods or the property of the M2 enzyme to be inhibited by ATP [20] or L-alanine [19]. The M2-specific antipeptide antiserum may enable us to determine the expression of PK isozymes in individual cells during development and carcinogenesis.

In this report we used a synthetic peptide for the production of specific antibodies. Compared with the production of monoclonal antibodies, this method has the advantage that specific regions of interest can be chosen from the protein concerned beforehand, while the resulting antibodies will in fact have a 'monoclonal' character.

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