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Urinary Adenylate Kinase Isoenzyme Pattern in Patients with Myocardial Infarction

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In memory of the late professor Gunnar Ågren

ABSTRACT

Adenylate kinase was purified in pooled urinary samples from patients with uncomplicated myocardial infarction. The purification procedure included ammonium sulfate precipitation and column chromatographic steps. It was necessary to stabilize the enzyme during purification with 2-mercaptoethanol and AMP. Polyacrylamidgelelectrophoresis in sodium dodecanyl sulfate revealed the release of 4 different isoenzymes of AK into urine from patients with myocardial infarction. The molecular weights of these isoenzymes were estimated to be 21,000; 24,000; 33,000 and 36,000, respectively.

INTRODUCTION

The measurement of serum enzyme activities is an established laboratory procedure supplemental to clinical and electrocardiographic examination for diagnosis of myocardial infarction. The most widely used enzymes in this context include ASAT [8], LD [17] and CK [6]. Since difficulties have arisen in finding an enzyme specific enough for the myocardium, several different enzymes appearing in the serum have been used as a diagnostic aid for establishing myocardial infarction [2,16].

AK was found in elevated amounts in serum during the early phase of myocardial infarction [7]. Different reports on the molecular weight of AK have been given. Hence the molecular weight of rabbit muscle AK was 21,000 while that of Baker's yeast was 41,000 [4]. Due to the low molecular weight of the enzyme it was established to be present also at a high rate in urine in connection with myocardial infarction [7].

The present communication deals with the purification of AK from large amounts of pooled urines from patients with uncomplicated myocardial infarction.

MATERIAL AND METHODS

Abbreviations. Adenylate kinase (AK), alanine aminotransferase (ALAT), aspartate amino transferase (ASAT), creatine kinase (CK), diethyl aminoethyl (DEAE), ethylene diamine tetraacetic acid (EDTA), lactate dehydrogenase (LD), polyacrylamidegelelectrophoresis (PAGE), rotations per minute (r p m), sodiumdodecanylsulfate (SDS), Tris(hydroxymethyl)-aminoethane (Tris), ultraviolet (UV).

Collection of urine. Urine was collected during the first 3 days after admission to the hospital.

The diagnosis of myocardial infarction was based upon the following three criteria.

a) Clinical history - typical clinical history with acute central chestpain of at least 15 min duration.
b) Positive electrocardiographic findings - the appearance of a Q-wave and/or S-T elevations followed by subsequent T-inversions in a localized area.
c) Positive serum enzyme pattern - typical enzyme pattern with an increase of ASAT with lower ALAT and/or increase of LD in a characteristic time course.

At least two of these three criteria should be fulfilled. Every effort was performed to rule out irrelevant diseases such as pulmonary embolism, cardiac congestion or liver disease.

The urine was voided into plastic or glass bottles and immediately put into refrigerator. Dithiothreitol was added to each bottle as an thiolgroup reactant giving a final concentration of 1 mM in the urine.

The urine was examined for presence of blood and albumin and if such a contamination was found, the urine was discarded. The urine specimens were kept cool until further handling in the laboratory, which took place within 24 h. Only urine that contained AK activity was considered for further handling. They were kept frozen for at most 3 months until use.

The different lots of urine totally amounting to about 10 l were thawed over night in room temperature. They were filtered once and concentrated about 25 times by ultrafiltration [1] . The concentrated urine thus obtained was centrifuged for 30 min in a Sorvall refrigerated centrifuge using Rotor number SS-34 at 6000 rpm resulting in a small pellet that was discarded. The clear supernatant usually amonting to about 400 ml contained about 5 mg nitrogen/ml micro-Kjeldahl method).

Purification of the enzyme from urine:

1. Precipitation with ammoniumsulfate. Ammonium sulfate at 0.4 saturation was added and the urine was kept overnight at 4^{o}C.The urine was centrifuged for 60 min at 4^{o}C and at 5000 rpm in the Sorvall centrifuge. The precipitate

was washed once in ammoniumsulfate at 0.4 saturation and the two supernatants were combined. A second precipitation was performed with ammonium sulfate at 0.8 saturation and kept overnight at 4°C. Centrifugation followed as above and the precipitate was washed once in ammonium sulfate at 0.8 saturation. The precipitate was dissolved in 10 ml of 0.5 M $NaHCO_3$, pH 7.1, and dialysed for 4 h with two changes of external water that contained 5 mM $MgCl_2$ and the 2-mercaptoethanol.

2. Chromatography on Blue Sepharose Cl-6B (Pharmacia, Uppsala, Sweden). All separatory steps were performed at 4°C. The Blue Sepharose was equilibrated with 0.02 M TrisHCl, pH 7.5 containing 5mM $MgCl_2$ and 0.4 mM EDTA and 2 µM 2-mercaptoethanol. About 90 ml of the buffer-equilibrated gel was used each time.

The ammonium sulfate-fractioned urinary material representing an amount of about 365 mg was applied to the Blue Sepharose column. Elution started with the 0.02 M TrisHCl-buffer containing Mg^{2+}, EDTA and 2-mercaptoethanol. AMP was added to a final concentration of 5 mM. The next step included the addition of 0.2 M NaCl and the final one, 0.4 M NaCl (Fig. 1). No additional activity was recovered when increasing NaCl conc to 0.8 M. The fraction containing AK activity of Fig 1 was concentrated by ultrafiltration and dialysed (5 mM $MgCl_2$ and 2 µM 2-mercaptoethanol) before freeze-drying.

3. Fractioning on DEAE-Sephacel column (Pharmacia, Uppsala, Sweden). The main fraction containing AK activity of figure 1 was subjected to further purification on DEAE-Sephacel. About 300 mg of the lyophilized material was applied to a 50 ml column of DEAE-Sephacel by dissolving it in 3 ml of a 0.015 M TrisHCl buffer pH 8.0 containing 2 µM 2-mercaptoethanol and 5 mM AMP. Gradient elution was carried out by mixing 260 ml 0.015 M Tris HCl pH 8.0 containing 2-mercaptoethanol and AMP with 260 ml of the same buffer also containing 1M KCl. Three UV-absorbing peaks were identified, the third of which also contained AK-activity. This active material was dialysed against $MgCl_2$ and 2-mercaptoethanol and lyophilized as described before.

4. Gel chromatography. The lyophilized material amounting to about 225 mg was dissolved in 1.25 ml distilled water and applied to a 25 ml Sephadex G25 column and eluted with distilled water. Low molecular weight material (salts and AMP) was separated from AK.

5. Polyacrylamidgelelectrophoresis in sodiumdodecanylsulfate. 1 mg of purified material was dissolved in 0.5 ml of the gel buffer consisting of 7.8 g of $NaH_2PO_4 \cdot H_2O$; 25.63 g of $Na_2HPO_4 \cdot 2H_2O$; 2 g of SDS dissolved in 1 litre distilled water. PAGE in the presence of SDS was in accordance with Shapiro et al [12].

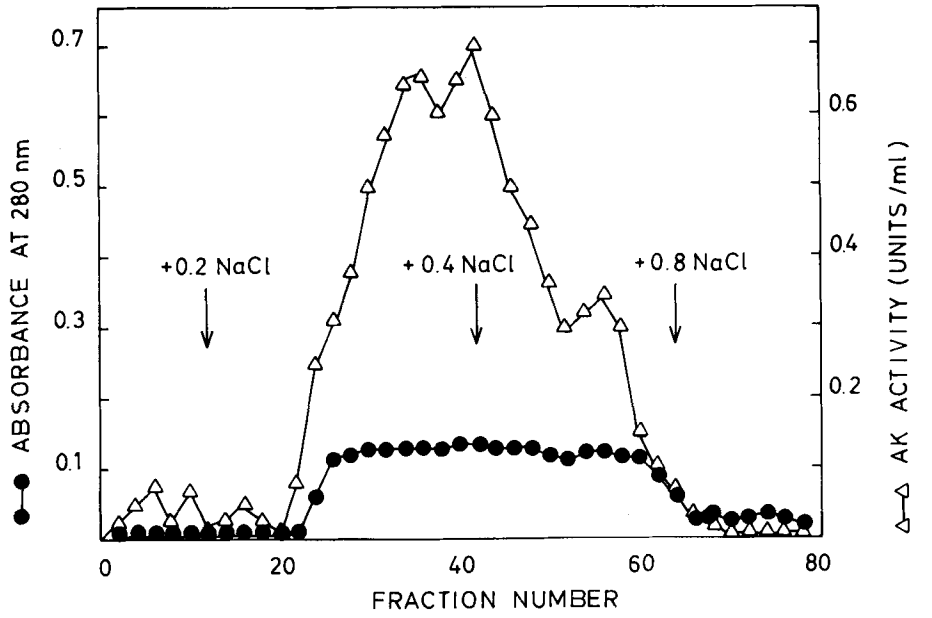

Fig 1. Chromatography of ammonium sulfate-fractionated human urinary material on Blue Sepharose. Material representing an amount of about 360 mg was applied to the column containing 90 ml of buffer-equilibrated gel. Elution started with the buffer that was 0.02 M Tris-HCl, pH 7.5, containing 5 mM $MgCl_2$, 0.4 mM EDTA, 2 µM 2-mercaptoethanol and 5 mM AMP. Arrows in Figure indicate the additions of 0.2 M, 0.4 M and 0.8 M of NaCl, respectively, to the eluent buffer.

The electrophoretic mobilities of the AK isoenzymes were plotted against the logarithm of known protein-standard mixture II supplied by Merck Reagents (E. Merck AB, Stockholm, Sweden) following the technique described by Weber and Osborn [15].

AK activity in urine was determined at 25°C in accordance with a previous report [7].

RESULTS

The specific AK activity of concentrated, filtered and centrifuged urine was 0.45 units x mg N^{-1} x min^{-1} (25°C). This activity increased by about 4.5 times to 2.1 units x mgN^{-1} min^{-1} after the precipitation steps with ammonium sulfate and dialysis prior to separation on Blue Sepharose. The result of this column chromatography is given in Fig.1.

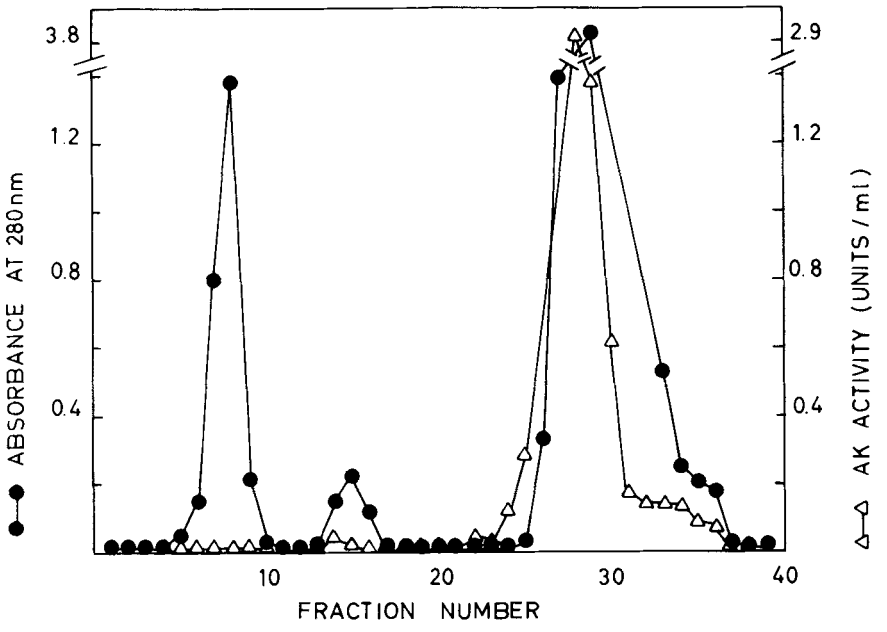

Fig 2. DEAE-Sephacel chromatography of urinary adenylate kinase recovered from purification on Blue Sepharose. About 300 mg of lyophilized material was applied to the 50 ml column of DEAE-Sephacel by dissolving it in 3 ml of 0.015 M Tris-HCl buffer, pH 8.0, containing 2 µM 2-mercaptoethanol and 5 mM AMP. Gradient elution carried out by mixing 260 ml of the Tris-buffer solution with an equal amount of the same solution also containing 1M KCl.

A broad UV-absorbing peak was detected by continuous reading at 280 nm. The AK activity was concentrated in this major fraction of the chromatogram (Fig 1). This fraction was subjected to further purification on DEAE-Sephacel (Fig 2). Three peaks with UV light absorption at 280 nm were recovered and practically all AK activity was found in the third peak. The material of this peak was gel-filtered on Sephadex G25 (Fig 3). The AK-bearing peak (first peak) was hereby separated from AMP (second peak). The material with AK activity recovered from Sephadex G25 column chromatography was concentrated and analysed on PAGE in the presence of 0.2% SDS. Four different bands were identified and the molecular weights were estimated as follows: Band 1, 21,000; band 2, 24,000; band 3, 33,000 and band 4, 36,000 (Fig 4).

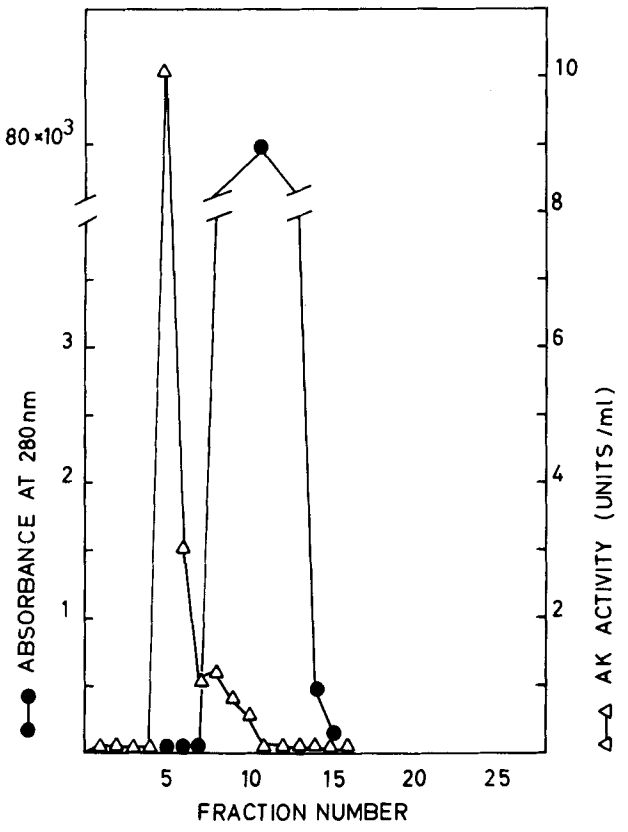

Fig 3. Chromatography of main peak with AK activity (Fig 2) on Sephadex G25. The lyophilized, active material, about 225 mg, was dissolved in 1.25 ml distilled water and applied to the column containing 25 ml of Sephadex G25. Elution was performed with distilled water.

DISCUSSION

We have isolated four different isoenzymes of AK in urine of patients with myocardial infarction. The molecular weights of the isoenzymes ranged between 21,000 - 36,000. Our results agree fairly well with those of Russell et al [10] who claimed that human heart tissue may contain five AK isoenzymes in the molecular weight range 21,000 - 31,000. Their results further indicated that AK isoenzymes may reflect three orders of specificity, namely a species specificity, an organ specificity and a subcellular specificity. Therefore the isoenzymes studied in the present work might all derive from the myocardial tissue but from different organelles as well as from the cytosol.

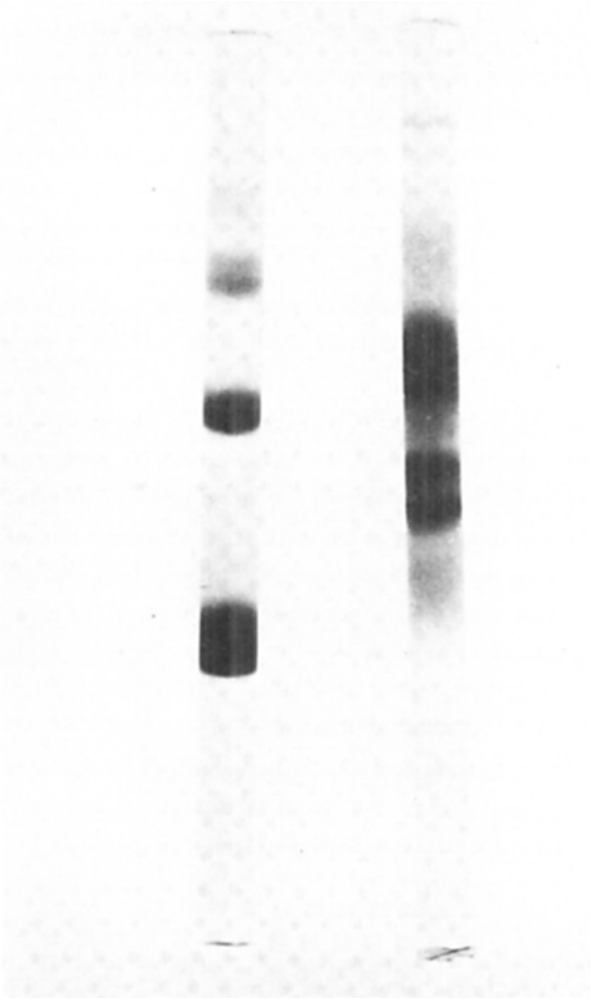

Fig 4. SDS-PAGE of 1 mg of purified material with AK activity. The gels show proteins with known molecular weights (left, Protein standard mixture II from Merck Reagents) and the different isoenzymes of active peak 1 (Fig 3) from Sephadex chromatography (right).

Since AK was isolated from urine initially during the course of myocardial infarction and only from patients displaying an uncomplicated picture of this acute heart disease, we have reasons to believe that the isoenzymes were not derived from other organs.

Further, for comparison, AK activities were measured in urines from one patient with uncomplicated myocardial infarction and another with advanced liver disease due to alcoholic abuse.

These measurements were performed in the absence and presence of $1x10^{-8}$M diadenosine pentaphosphate. An inhibitory action by the additive was typically registered in the urine from the patient with myocardial infarction. Such a pattern was not observed in the other patient with liver disease, here instead a slight stimulation was apparent (Data not given in Results). (cf.ref 11). Hence, the patient with uncomplicated heart disease displayed an urinary enzyme with different characteristics vis à vis the inhibitor compared to the patient with liver disease.

The isoenzymes studied most probably appear as single polypeptide chains [14] and not in a hybrid dimeric form similar to CK [3] or a tetrameric form like LD [5].

The enzyme was labile during purification and it was necessary to supply the suspension medium with AMP as well as thiol group reagents to stabilize the enzyme. Nevertheless a certain degree of inactivation probably took place during purification (cf.ref 9,13). By large scale preparation of purified AK from urine of patients with myocardial infarction, enough material has been obtained for the purpose of antibody production for use as a diagnostic tool. Such work is under progress.

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