*Biochimica et Biophysica Acta*, 480 (1977) 521—526


BBA Report

BBA 61314

# RADIOIMMUNOASSAY FOR CANINE CREATINE KINASE ISOENZYMES

ROBERT ROBERTS and AUDREY PAINTER

*Department of Cardiology, Washington University School of Medicine, St. Louis, Mo. (U.S.A.)*

(Received September 27th, 1976)

## Summary

Antibodies specific for the B and M subunits of creatine kinase (ATP: creatine *N*-phosphotransferase, EC 2.7.3.2) isoenzymes were obtained by immunization of rabbits with canine BB and MM creatine kinase. A radioimmunoassay capable of measuring picomolar amounts of creatine kinase isoenzymes has been developed which measures concentrations of intact isoenzymes rather than activity. It should aid in elucidating the metabolism and breakdown of creatine kinase isoenzymes.

Detection of organ injury and other abnormalities is often based on elevation of appropriate enzyme or isoenzyme activity in the circulation. In spite of the widespread diagnostic use of plasma enzymes, very little is known of their precise fate in the circulation, or factors influencing their rates of disappearance from the circulation [1]. Furthermore, it is not clear whether disappearance of enzyme activity is rate-limited by inactivation, denaturation, or removal of intact enzyme molecules from the circulation. This is, in part, related to the lack of an assay to measure the concentration of intact isoenzyme molecules, since present assays detect activity rather than enzyme protein concentration.

Creatine kinase (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2) exists in at least three isoenzyme forms (each with molecular weight of approximately 82 000) designated MM, MB, BB on the basis of subunit compositions. The only human tissue containing appreciable amounts of MB is myocardium, and elevated plasma MB activity is a remarkably sensitive and specific marker of myocardial injury [2, 3]. In contrast, canine myocardium contains only MM (97—99%), and its elevation in the plasma after myocardial injury is a highly sensitive diagnostic index [4]. Analysis of serial changes in plasma creatine kinase activity has been utilized to quantify the amount of myocardial damage

Please address correspondence to: Robert Roberts, M.D., Director, Cardiac Care Unit, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, Mo. 63110, U.S.A.

(infarct size) associated with myocardial infarction in experimental animals [4], and patients [5]. However, these estimates are directly dependent on the rate of disappearance of creatine kinase from plasma [1]. Despite creatine kinase isoenzymes having similar molecular weight, specific substrate and other properties, their rates of disappearance from the circulation is strikingly different and not directly related to lability in solution in vitro [6]. Similar observations have been made for lactate dehydrogenase isoenzymes [7].

Radioimmunoassays used to assay hormones in the circulation have significantly advanced our knowledge of metabolism. Application of radioimmunoassay to isoenzymes has not been possible, in part because of difficulty in labelling with high specific radioactivity due to loss of enzyme activity and dissociation of subunits upon exposure to oxidizing agents in conventional labelling procedures. The present study was designed to develop a radioimmunoassay for canine plasma creatine kinase isoenzymes which would determine the plasma enzyme protein concentration, and help to characterize the nature of creatine kinase disappearance from the circulation, and facilitate detection and quantification of myocardial infarction.

Creatine kinase isoenzymes were purified from canine tissues removed immediately after killing the animal. MM isoenzyme was purified from canine myocardium, MB from small intestine, and BB from human brain as recently described [8]. Tissues were minced and homogenized in 0.05 M Tris·HCl, pH 7.4, containing 0.001 M 2-mercaptoethanol, centrifuged at 31 000 x *g*, and extracted repetitively with ethanol. The residue containing the creatine kinase isoenzymes was precipitated with 70% ethanol, resuspended, and isoenzymes separated completely from each other by batch adsorption and column chromatography, with DEAE-Sephadex A50, dialyzed, freeze-dried, and stored at 4°C.

Canine MM (1 mg) and canine BB creatine kinase (1 mg) mixed with equal volumes of Freund's complete adjuvant were injected subcutaneously (0.25 mg/foot pad) into rabbits and the serum harvested for antibodies to canine creatine kinase isoenzymes ten days after monthly booster injections of 0.1 mg. Ouchterlony agarose plates, prepared with the MM antiserum, exhibited a single precipitant line to MM and MB antigens, but no precipitant line to BB. Plates prepared with BB antiserum exhibited a single precipitant line to both MB and BB but none to MM.

Introduction of $^{125}I$ into the isoenzyme molecule by the chloramine-T or lactoperoxidase methods resulted in marked loss of enzyme activity, possibly due to oxidation of sulfhydryl groups. To avoid exposing the isoenzymes to oxidizing agents and contaminants in the radioiodine, the $^{125}I$ (5 mCi) was first incorporated into the N-succinimidyl 3-(4-hydroxyphenyl)propionate (0.3 μg) according to the method of Bolton and Hunter [9]. The labelled ester was extracted into benzene, recovered by evaporation and the iodinated residue combined with MM, MB, or BB creatine kinase (1–2 mg) in 1–2 ml of 0.01 M sodium borate buffer, pH 8.5. After shaking the reaction mixture for 4 h at 4°C, the labelled isoenzyme was then dialyzed against the same buffer containing 0.002 M 2-mercaptoethanol resulting in specific radioactivity for MM and MB creatine kinase of 0.12 μCi/μg of protein and for BB 0.06 μCi/μg, with less than 5% loss of enzyme activity.

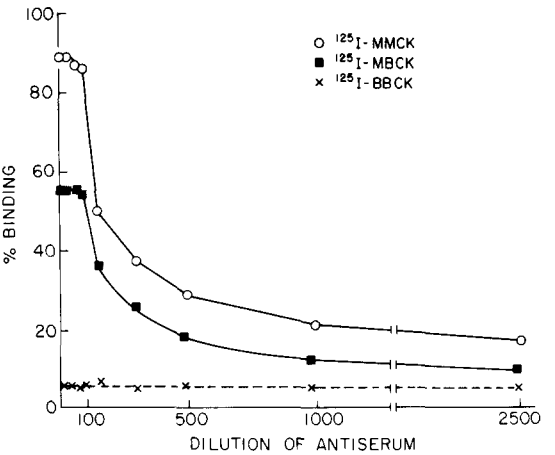


**Fig. 1. Specificity of MM antiserum for M subunit. The figure shows the specificity of MM antiserum for MM and MB. MM antiserum binds $^{125}$I-labelled MM and $^{125}$I-labelled MB which is dependent on the concentration of MM antibody. There is no binding of $^{125}$I-labelled BB creatine kinase (CK) at any concentration of MM antibody.**

To determine the binding affinity of antibody to creatine kinase isoenzymes, radioiodinated canine BB, MB, and MM creatine kinase were incubated with MM antiserum, diluted 1 : 10 to 1 : 2500, and antibody-bound $^{125}$I-labelled antigen was separated from free $^{125}$I-labelled antigen by ammonium sulphate precipitation [10], and binding was determined by the amount of $^{125}$I activity in the pellet. Using MM and MB as antigens, maximal binding was 90% and 56% respectively but binding to BB was only 3—5% which was similar to control (Fig. 1). This would suggest that the antibodies were directed toward the respective subunits rather than the molecule as a whole. MM antiserum in a dilution of 1 : 10 bound 90% of the $^{125}$I-labelled MM as shown by the curve, but bound much less (22%) at a dilution of 1 : 1000, indicating that the binding is dependent on the concentration of antibodies. Similarly, concentration dependent binding was demonstrated for MB, but such was not the case with BB. Binding to BB was consistently 3—5% which was similar to control and thus nonspecific. The converse result was obtained with BB antiserum with 93% and 56% binding of the labelled BB and MB respectively, and only 2—3% binding of the MM. With all three antigens, normal rabbit serum produced only 2—3% binding. Thus, binding of the radioiodinated antigens was immunologically specific, and there was no apparent cross-reactivity between antibodies to the B and M subunits.

To determine whether the MM antiserum could be used in a competitive displacement binding radioimmunoassay, and its specificity in this system, it was necessary to compare the ability of unlabelled MM, MB and BB creatine kinase to inhibit $^{125}$I binding. All determinations performed in triplicate were carried out in 12 x 75 mm glass tubes containing 1.6 M Tris buffer, pH 7.6, (200 $\mu$l), 2% bovine serum albumin (100 $\mu$l), 0.020 M mercaptoethanol (10 $\mu$l), 5 pg of rabbit $\gamma$-globulin (50 $\mu$l). To this was added 1 : 150 dilution of MM antiserum, and $^{125}$I-labelled MM which was kept constant at 0.15 mcg containing approximately 25 000 cpm. The antiserum dilution of 1 : 150 was chosen since

this concentration of antibody binds about 50% of the $^{125}$I-labelled MM. A known amount of unlabelled MM, MB, or BB creatine kinase was diluted from 1:15 to 1:1000 and incubated for four hours with the $^{125}$I-labelled MM creatine kinase. Following incubation, the ammonium precipitated pellet was washed, centrifuged and counted for $^{125}$I radioactivity. To further determine the specificity of unlabelled MM or MB to displace $^{125}$I-labelled MM binding in the face of BB creatine kinase, inhibition curves were determined for MM or MB in which BB was present in a 10 000-fold excess over that of unlabelled MM or MB. MM and MB produced a concentration related diminution of $^{125}$I binding with 50% inhibition at 5 and 11.2 ng respectively, and essentially complete inhibition of binding at concentrations of 100 ng/ml and above (Fig. 2). With BB creatine kinase, no inhibition of $^{125}$I-labelled MM binding occurred, even at 1 mg/ml (a 10 000-fold molar excess over $^{125}$I-labelled MM).

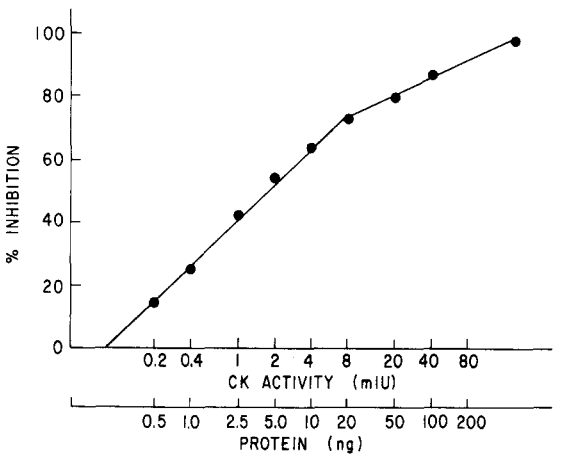


**Fig. 2. Competitive displacement by MM creatine kinase. The figure shows competitive displacement of $^{125}$I-labelled MM creatine kinase by unlabelled MM creatine kinase (CK), which is concentration-dependent. The inhibition curve is steep in the range of 0.5—80 ng.**

Thus, in the MM system the competitive displacement assay for MM is not only very sensitive but can detect small quantities of MM in the presence of much larger quantities of BB. A known amount of purified dog heart MM was added to dog serum and diluted over a range of 1:10 to 1:1000. Determinations of enzyme protein by the radioimmunoassay deviated from that expected by less than 5% and was unaffected by the addition of purified dog brain BB creatine kinase. Results on blood samples obtained from a dog during myocardial infarction after coronary occlusion are shown in Fig. 3.

The present study describes the development of a radioimmunoassay for plasma creatine kinase isoenzymes which is extremely sensitive (picomolar amounts) based on antibodies specific for either the M or B subunit. Labelling the isoenzymes via the iodinated succinimidyl ester produced adequate specific radioactivity with minimal loss of enzyme activity which is not possible in conventional methods. Though the specific radioactivity was low compared to that used in most radioimmunoassays, it was more than adequate in terms of sensitivity since one can detect picogram amounts and normal levels of

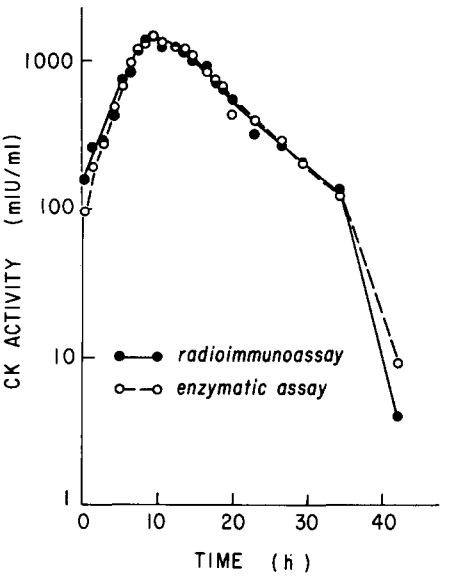


Fig. 3. Plasma creatine kinase activity after infarction. A plasma creatine kinase (CK) time activity curve in a dog with myocardial infarction. Since 1 ng of purified MM enzyme protein was equivalent to 0.4 mI.U. of enzyme activity, values obtained with the radioimmunoassay method expressed initially as ng/ml, were converted to enzymatic activity as shown with the solid circles. Results were compared to those obtained by a kinetic fluorometric assay which measures enzymatic activity, shown with open circles. As can be seen, values obtained with the two methods are in close agreement.

creatine kinase are 10—20 ng/ml. Higher specific radioactivity can be obtained by increasing the ratio of $^{125}I$ to protein as originally described by Bolton and Hunter [9]. However, with adequate sensitivity, we felt it was an advantage to have a low iodine substitution with high antigenic binding providing for a short incubation period of four hours as opposed to the usual 12—48 h. Stabilizing the molecule with 0.020 M of 2-mercaptoethanol and 1.6 M Tris prevents dissociation of subunits and consistent results in separating bound iodinated antigen from free iodinated antigen. This approach may be applicable for development of radioimmunoassay for other plasma isoenzymes. Since this assay detects the concentration of molecules, one can determine the actual rate of enzyme turnover independent of enzyme activity and should help in elucidating mechanisms responsible for disappearance of individual creatine kinase isoenzymes from the circulation as well as relative importance of inactivation, denaturation, or actual removal of molecules.

Of equal importance, because of its sensitivity and potential for detecting enzymatically inactive creatine kinase isoenzymes in the circulation, the creatine kinase radioimmunoassay may lead to improved enzymatic estimate of infarct size as well as earlier detection of acute myocardial infarction. Although several methods for assaying creatine kinase isoenzyme activity have been developed [11—13], they are not ideally suited for quantitative analysis of large numbers of samples, their specificity is somewhat limited; and they rely exclusively on detection of enzyme activity. Conventional enzymatic assays can barely detect 0.01 I.U./ml whereas the present assay reliably detects 0.0002 I.U./ml. Early detection of myocardial infarction is of particular im-

portance today in view of the recent enthusiasm to protect ischemic myocardium since agents which could potentially decrease infarct size would be more effective if administered early.

This work was supported in part by a SCOR grant from the National Heart and Lung Institute. We thank Carole Goodell for her assistance.

## References

1 Roberts, R., Henry, P.D. and Sobel, B.E. (1975) Circulation 52, 743—754
2 Roberts, R., Gowda, K.S., Ludbrook, P.A, and Sobel, B.E. (1975) Am. J. Cardiol. 36, 433—437
3 Konttinen, A, and Somer, H. (1972) Am. J. Cardiol. 29, 817—820
4 Shell, W.E., Kjekshus, J.K. and Sobel, B.E. (1971) J. Clin. Invest. 50, 2614—2625
5 Sobel, B.E., Bresnahan, G.F., Shell, W.E. and Yoder, R.D. (1972) Circulation 46, 640—648
6 Robison, A.K., Gnepp, D.R. and Sobel, B.E. (1975) Circulation Suppl. II, 52, 5
7 Boyd, J.W. (1967) Biochim. Biophys. Acta 132, 221—231
8 Carlson, E., Roberts, R. and Sobel, B.E. (1976) J. Mol. Cell. Cardiol. 8, 159—167
9 Bolton, A.E. and Hunter, W.M. (1973) Biochem. J. 133, 529—539
10 Farr, R.S. (1958) J. Infect. Disord. 103, 239—262
11 Roberts, R., Henry, P.D., Witteveen, S.A.G.J. and Sobel, B.E. (1974) Am. J. Cardiol. 33, 650—654
12 Henry, P.D., Roberts, R. and Sobel, B.E. (1975) Clin. Chem. 21, 844—849
13 Mercer, D.W. (1974) Clin. Chem. 20, 36—40