Journal of Chromatography, 205 (1981) 213–217
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,309

Note

Chromatography of kidney propionyl-CoA and 3-methylcrotonyl-CoA carboxylases on Cibacron Blue- and Procion Red-substituted agaroses

EDWARD P. LAU and R. RAY FALL*
Department of Chemistry, University of Colorado, Campus Box 215, Boulder, CO 80309 (U.S.A.)
(First received April 22nd, 1980; revised manuscript received September 1st, 1980)

Propionyl-CoA carboxylase (PCase) and 3-methylcrotonyl-CoA carboxylase (MCase) are biotin-containing enzymes that catalyze the ATP-dependent carboxylation of their respective acyl-CoA substrates[1]. Recently, we purified both PCase and MCase from bovine kidney mitochondria[2]. Since the two enzymes are very similar in molecular weight and isoelectric point, and co-purify in a variety of chromatographic procedures, it is a tedious process to separate them from one another by conventional techniques. This communication describes simple procedures using affinity chromatography on Cibacron Blue- and Procion Red-substituted agaroses to completely resolve the two enzymes and provide more facile purification of each. These procedures should be of value to workers attempting to isolate PCase and MCase from human tissues from patients with genetically inherited defects in PCase or MCase[3–5].

MATERIALS AND METHODS

Materials

Blue Dextran Sepharose 4B was prepared according to published procedures[6], except that only 0.5 g Blue Dextran 2000 (Pharmacia, Uppsala, Sweden) was used. Blue Sepharose CL-6B (2.5 μmole chromophore/ml) and DEAE-Sephacel were purchased from Pharmacia. Reactive Blue 2-agarose (3.4 μmole chromophore/ml) and Reactive Red 120-agarose (2.5 μmole chromophore/ml) were from Sigma (St. Louis, MO, U.S.A.). The sources of other materials used have been described[2,7].

Enzyme assay

PCase and MCase were assayed by a $^{14}CO_2$ fixation procedure as described[7], except that both enzymes were assayed in the presence of 100 m*M* potassium chloride. Protein was determined by a dye binding method using crystalline bovine serum albumin as standard[8].

Enzyme purification

Frozen diced bovine kidney (320 g) was passed through a meat grinder twice and homogenized in a Waring blender for 2 min in 500 ml 20 m*M* potassium phosphate, pH 7.0, containing 0.1 m*M* EDTA and 0.1 m*M* dithiothreitol (buffer K). Centrifugation of the homogenate at 48,000 *g* for 20 min yielded a supernatant solu-

0021-9673/81/0000–0000/$02.50 © 1981 Elsevier Scientific Publishing Company

tion containing the majority of the total PCase and MCase activity. (Separate experiments have shown that the MCase present in the 48,000 *g* supernatant fraction is a mixture of "solubilized" enzyme and enzyme bound to mitochondrial membrane fragments; see ref. 9.) To this solution was added slowly 50% (w/v) polyethylene glycol 6000 (PEG 6000, Sigma) to give a final concentration of 3% (w/v) PEG 6000. After stirring for an additional 10 min, the suspension was centrifuged at 18,000 *g* for 15 min. The pellet contains less than 10% of the PCase but approximately half of the MCase and can be used for the purification of MCase[10]. The supernatant, containing the remainder of the PCase and MCase activities, was used for the studies described here. One third of the supernatant was applied to a DEAE-Sephacel (Pharmacia) column (15 × 3 cm) equilibrated with buffer KG (buffer K in 10% (v/v) glycerol. The column was washed with 150 ml buffer KG. A linear gradient of 0–0.3 *M* potassium chloride in buffer KG was used to elute the enzymes; PCase and MCase co-eluted at approximately 0.12 *M* KCl. The fractions containing PCase and MCase activity were pooled, concentrated and dialyzed against buffer KG, and then used for affinity chromatography studies.

Affinity chromatography

Affinity chromatography was performed on columns containing 5 ml of gravity-packed agaroses. These columns were equilibrated with buffer KG. Aliquots (3.7 mg protein in 0.1 ml) of the pooled, concentrated preparation from the DEAE-Sephacel column were applied to the columns, and the columns were sequentially washed with 15–20-ml aliquots of buffer KG or buffer KG plus supplements as described below. After chromatography, the columns were regenerated by washing with 50 ml of 5 *M* urea in buffer KG.

RESULTS AND DISCUSSION

We have previously outlined the isolation of bovine kidney MCase and PCase by conventional protein purification techniques starting with isolated mitochondria[2]. Subsequently, we have found that frozen bovine kidney is a more convenient starting material for the large scale isolation of these enzymes, and the isolation of mitochondria can be avoided. Fractionation of a kidney extract with polyethylene glycol yields a preparation of PCase and MCase that can be directly chromatographed on DEAE-Sephacel. The DEAE-Sephacel step is useful for removing proteins that interfere with the subsequent affinity chromatography, but does not resolve PCase and MCase which co-elute even when a shallow potassium chloride gradient is employed.

In order to further purify and resolve PCase and MCase we turned to the use of dye-substituted agaroses that have recently been used to advantage for the affinity chromatography of nucleotide-dependent enzymes including various ATP-utilizing enzymes (see ref. 11). We tested three commercially available dye-substituted agaroses: (1) Blue Sepharose, a cross-linked agarose containing the dye Cibacron Blue F3G-A linked through the triazine ring[12], (2) Reactive Blue-2 agarose, similar to Blue Sepharose except the dye is linked to a non crossed-linked agarose and (3) Reactive Red-120 agarose, containing the dye Procion Red HE-3B linked to a cross-linked agarose matrix through the triazine ring[13]. We also prepared Blue Dextran agarose as previously described[6]; in this case the dye Cibacron Blue F3G-A linked to Dextran-

2000 (Pharmacia) via the triazine ring is coupled to a cyanogen bromide activated agarose presumably by reaction with the free amino group of the dye (see ref. 12).

The partially purified enzyme preparation containing PCase and MCase was passed through the four different dye-substituted agarose columns and various elution conditions were explored. The results are shown in Tables I and II. Both enzymes were readily adsorbed on all four dye-substituted agaroses. Using ATP at concentrations of 1–3 m*M* to effect elution, PCase activity was eluted only from the Blue Dextran Sepharose and Blue Sepharose columns. No PCase activity was eluted from the Reactive Blue- or Reactive Red-agarose columns even when 3 m*M* ATP was used. The highest increase in specific activity for PCase was attained by using a 1 m*M* ATP wash of the Blue Sepharose column (Table I). No MCase activity could be recovered from any of the columns using ATP elution.

Attempts to elute adsorbed PCase and MCase using salt elution are summarized in Table II. PCase could be eluted from all the columns using varying concentrations

TABLE I

ATP ELUTION OF CARBOXYLASE ACTIVITIES FROM AFFINITY COLUMNS

Columns	*Activity of total applied* (%)*					
	KG wash		*1 mM ATP in KG wash*		*3 mM ATP in KG wash*	
	MCase	*PCase*	*MCase*	*PCase*	*MCase*	*PCase*
Blue Dextran Sepharose	0	15 (5.7)	0	30 (41.7)	0	30 (81.8)
Blue Sepharose	0	15 (5.7)	0	130 (344.1)	0	12 (52.9)
Reactive Blue Agarose	0	0	0	0	0	0
Reactive Red Agarose	0	0	0	0	0	0

* Values in parentheses are specific activities in nmole/min/mg protein. Enzyme activity was determined by the $^{14}CO_2$-fixation method. The specific activites of the applied enzyme preparation were: PCase, 28.0; MCase, 3.6.

TABLE II

KCl ELUTION OF CARBOXYLASE ACTIVITIES FROM AFFINITY COLUMNS

Columns	*Activity of total applied* (%)*							
	KG wash		*0.1 M KCl in KG wash*		*0.3 M KCl in KG wash*		*1.0 M KCl in KG wash*	
	MCase	*PCase*	*MCase*	*PCase*	*MCase*	*PCase*	*MCase*	*PCase*
Blue Dextran Sepharose	0	15 (5.7)	0	110 (81.8)	66 (16.8)	21 (42.4)	8 (3.4)	3 (12.4)
Blue Sepharose	0	15 (7.7)	0	71 (67.7)	18 (2.3)	40 (40.4)	21 (3.0)	2 (2.6)
Reactive Blue Agarose	0	0	0	0	0	12 (8.1)	0	59 (34.6)
Reactive Red Agarose	0	0	0	0	0	43 (31.1)	0	138 (102.0)

* See footnote to Table I.

of potassium chloride, although recovery from Reactive Blue-agarose was poor. MCase could only be eluted in high yield from Blue Dextran Sepharose using 0.3 *M* potassium chloride; no MCase could be eluted from Reactive Blue- or Reactive Red-agarose even when 1 *M* potassium chloride was used.

In general MCase was more tightly adsorbed than PCase by all the agaroses. In the cases of Reactive Blue-agarose and Reactive Red-agarose we were unable to recover (elute) any MCase under conditions employed, including washing with combinations of salts and ATP. Extensive pre-washing of the columns or dilution of the dye-substituted agaroses with unsubstituted ones did not help in this regard. The reason for this tight binding is unclear. It may be due to the method of preparation of these columns, or differences in cross-linking. The lower affinity binding of MCase (and PCase) to Blue Dextran Sepharose than to Blue Sepharose is analogous to previous results obtained with phosphofructokinase[11].

The usefulness of Blue Sepharose for the resolution and further purification of PCase and MCase is shown in Fig. 1. An aliquot of the DEAE-Sephacel pool was applied and a combination of ATP and salt elution was utilized. PCase free from MCase was eluted by 3 m*M* ATP in >85% yield with a purification factor of approximately 15 in this step. All the MCase activity of the preparation remained adsorbed to the column under these conditions, and also when some PCase and protein was eluted with 0.1 *M* KCl (Fig. 1). MCase free from PCase was eluted by 1 *M* KCl plus 1 m*M* ATP in >70% yield with a purification factor of approximately 6; the addition of ATP to the 1 *M* potassium chloride eluting buffer improved the recovery of the enzyme. Gradient elution at this step might yield greater purification of MCase but has not yet been tested.

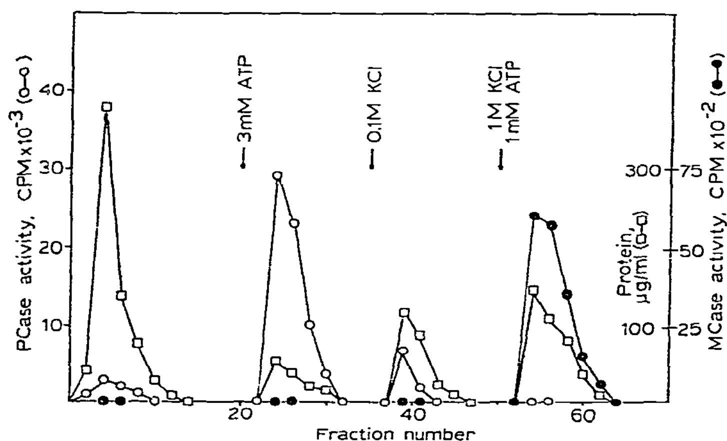

Fig. 1. Resolution of PCase and MCase on a Blue Sepharose column. A 0.1-ml aliquot of the dialyzed enzyme solution (3.7 mg protein) was applied to a Blue Sepharose column (5 ml settled bed column) equilibrated with buffer KG. Elution was carried out with buffer KG and then as indicated by the arrows with buffer KG containing 3 m*M* ATP, or 0.1 *M* KCl, or 1 *M* KCl plus 1 m*M* ATP. Fractions of approximately 1 ml were collected. 50-µl aliquots were assayed for carboxylase activity by the $^{14}CO_2$ fixation procedure[7] using propionyl CoA (○—○) or 3 methylcrotonyl CoA (●—●) as substrate. Protein (□—□) was determined by a dye binding procedure[8]. The specific activities (nmol/min/mg protein) of the applied enzyme preparation were: PCase, 24.2; MCase, 3.9. The specific activity of PCase in the eluted 3 m*M* ATP pool was 420 while that of MCase in the 1 *M* KCl plus 1 m*M* ATP pool was 23.4.

We have not used Blue Sepharose for the preparative isolation of PCase or MCase, but while this work was in progress we learned of the use of Blue Sepharose in the isolation of human liver PCase; in that case the enzyme was also eluted with ATP[14]. These results suggest that Blue Sepharose may be of general utility in the isolation of PCase from mammalian sources.

We have recently reported on the preparative isolation of bovine kidney MCase, using a purification procedure that included chromatography on Blue Dextran Sepharose[10]. Equally good or better purification of MCase can be achieved on the commercially available Blue Sepharose (as in Fig. 1), avoiding the need to synthesize Blue Dextran Sepharose. In addition, Blue Sepharose appears to be more stable upon repeated use, and does not lose bound Cibacron Blue dye as readily as Blue Dextran Sepharose.

The observations described in this communication may be of value to workers attempting the isolation of PCase or MCase from frozen human tissue or tissue taken at autopsy, where intact mitochondria are difficult to isolate[15], and where rapid affinity chromatography techniques can be very useful.

ACKNOWLEDGEMENT

This research was supported by a grant from the National Institutes of Health (HL 16628).

REFERENCES

1 H. G. Wood and R. E. Barden, *Annu. Rev. Biochem.*, 46 (1977) 385.
2 E. P. Lau, B. C. Cochran, L. Munson and R. R. Fall, *Proc. Nat. Acad. Sci. U.S.*, 76 (1979) 214.
3 W. Weyler, L. Sweetman, D. C. Maggio and W. L. Nyhan, *Clin. Chim. Acta*, 76 (1977) 321.
4 B. Wolf, *Biochem. Genet.*, 17 (1979) 709.
5 Y. E. Hsia, K. J. Skully and L. E. Rosenberg, *Pediatr. Res.*, 13 (1979) 746.
6 L. D. Ryan and C. S. Vestling, *Arch. Biochem. Biophys.*, 160 (1974) 279.
7 R. R. Fall and M. L. Hector, *Biochemistry*, 16 (1977) 4000.
8 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
9 M. L. Hector, B. C. Cochran, E. A. Logue and R. R. Fall, *Arch. Biochem. Biophys.*, 199 (1980) 28.
10 E. P. Lau and R. R. Fall, *Arch. Biochem. Biophys.*, 205 (1980) in press.
11 L. A. Haff and R. L. Easterday, in P. V. Sundaram and F. Eckstein (Editors), *Theory and Practice in Affinity Techniques*, Academic Press, London, 1978, p. 23.
12 H.-J. Böhme, G. Kopperschläger, J. Schulz and E. Hofmann, *J. Chromatogr.*, 69 (1972) 209.
13 D. H. Watson, M. J. Harvey and P. D. G. Dean, *Biochem. J.*, 173 (1978) 591.
14 F. Kalousek, M. D. Darigo and L. E. Rosenberg, *J. Biol. Chem.*, 255 (1980) 60.
15 A. J. Giorgio and T. R. Whitaker, *Biochem. Med.*, 7 (1973) 473.