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Radioimmunoassay of carbonic anhydrase III in rat tissues

Alan SHIELS,* Stephen JEFFERY,* Catherine WILSON† and Nick CARTER*‡
**Department of Child Health and †Department of Obstetrics and Gynaecology, St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, U.K.*

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1. A specific and sensitive radioimmunoassay for the rat carbonic anhydrase III isoenzyme was developed. 2. High concentrations of carbonic anhydrase III were detected in soleus muscle and male liver. 3. Female liver and other skeletal muscles contained significantly lower concentrations, and only trace amounts were found in heart, prostate, kidney, brain, plasma, urine and, possibly, erythrocytes.

In recent years high concentrations of a low-activity sulphonamide-resistant isoenzyme of carbonic anhydrase (carbonate dehydratase, EC 4.2.1.1) have been characterized from skeletal muscle of numerous mammals (Holmes, 1977; Register *et al.*, 1978; Carter *et al.*, 1979; Jeffery & Carter, 1980; Hewett-Emmett & Tashian, 1981). These proteins are homologous forms of an enzyme designated carbonic anhydrase III (CAIII) and were originally thought to be confined to red skeletal muscle. Nevertheless, trace amounts of CAIII have also been detected immunochemically in sheep lung, rabbit liver (Holmes, 1976) and human liver, smooth muscle, lung, heart (Jeffery *et al.*, 1980) and erythrocytes (Heath *et al.*, 1983). In the rat CAIII is also present in the liver (Carter *et al.*, 1981, 1982), where its expression is linked to age-dependent androgen control (Shiels *et al.*, 1983).

Radioimmunoassays have previously been described for monitoring the synthesis of the erythrocyte carbonic anhydrase isoenzymes (designated CAI and CAII) in patients of aberrant thyroid status (Spencer & Peller, 1976; Jeffery & Spencer, 1978). In addition, plasma CAIII concentrations measured by radioimmunoassay have been employed as an index of muscle breakdown in patients suffering from Duchenne muscular dystrophy (Heath *et al.*, 1982). As part of an investigation of the hormonal control of mammalian carbonic anhydrase isoenzymes, we have developed a sensitive and specific radioimmunoassay method to determine the distribution of CAIII in various rat tissues.

Abbreviations used: CAI, CAII and CAIII, carbonic anhydrase I, II and III isoenzymes.

‡ To whom correspondence should be addressed.

Methods and materials

Tissue collection

Blood was obtained from Wistar rats by cardiac puncture (thorax open) or tail venepuncture and sequestered in Li–heparin tubes (Sterilin) on ice. (N.B.: it was important to minimize contamination of the blood with ruptured muscle.) Plasma was collected by centrifugation (2000*g* for 10min at 4°C) and stored at −70°C. The buffy layer was discarded, and erythrocytes were washed three times in iso-osmotic saline before lysis at 4°C for 30min with 2vol. of distilled water. Stroma-free haemolysates were prepared by centrifugation. Urine was collected over 24h without preservatives before the rats were killed.

Intact skeletal muscles were excised and homogenized (Ultra-Turrax) for 30–60s in distilled water. Cell debris was removed by centrifugation at 30000*g* for 20min at 4°C, and the supernatants were stored at −70°C.

Preparation of anti-CAIII serum

Rabbit antiserum to purified rat CAIII was prepared as described previously (Carter *et al.*, 1981, 1982) and stored at −70°C.

Preparation of radioiodinated CAIII

Purified rat CAIII was radioiodinated by conjugation-labelling with the use of a commercially prepared Bolton and Hunter reagent (Amersham International) as described by Heath *et al.* (1982).

Radioimmunoassay procedure

Samples were assayed directly for CAIII (i.e. without extraction) in duplicate and standards in triplicate, essentially as described by Heath *et al.* (1982).

Results

Radioimmunoassay characteristics

The products of radioiodination were separated by gel filtration on Sephadex G-75 into two distinct peaks, and those column fractions for use as tracer in the radioimmunoassay were selected by the criteria of maximum specific radioactivity and immunoprecipitability (in the presence of excess antibody). Fractions similar in these respects were pooled. The modified Bolton and Hunter method gave 10–30% incorporation of total radioactivity into CAIII protein, and the specific radioactivity of the radioiodinated CAIII tracer was in the region of 10 μCi/μg of protein. The dilution of tracer (15×10^3–20×10^3 c.p.m./100 μl) was chosen arbitrarily, and the maximum incorporation of radioiodinated CAIII tracer that could be immunoprecipitated by excess anti-CAIII serum, $B_{max.}$, varied from 35 to 46% of the total radioactivity (c.p.m.) (T) added.

The concentration of antiserum chosen for use in the assay was sufficient to bind approx. 50% of the maximum binding ($B_{max.}$), i.e. a working dilution of approx. 1 : 4000.

The sensitivity of the assay was investigated by constructing a series of standard curves, covering a wide range of concentrations of unlabelled CAIII with fixed antiserum concentration, under different incubation conditions.

It was found that assay sensitivity was more than doubled when CAIII standard and anti-CAIII serum were incubated for 48 h before incubation with radioiodinated CAIII tracer for a further 24 h compared with incubating all three components continuously for 72 h. Therefore the procedure of 'late addition' of tracer was adopted in the assay.

The sensitivity of the assay was also influenced by the precision of reproducibility of measurement of the difference in radioactivity between repeated determinations of incubations. Inter-assay variation of standards never exceeded 20%. Sample variation within assays was set at 10%, and duplicate or triplicate determinations exceeding this variation in displacement of radioactivity (c.p.m.) were re-assayed. Sample variation between assays was determined by using 'quality-control' samples and never exceeded 25%. Fig. 1 shows a typical standard curve for purified rat CAIII. The minimum detection limit of the assay was considered to be approx. 1 ng of CAIII/ml, i.e. a B/B_0 value of 90%, which in turn represented the start of the linear part of the standard curve (Fig. 1).

The specificity of the anti-(rat CAIII) serum for the CAIII antigen was investigated by constructing standard curves with fixed antiserum and tracer concentrations (defined above), covering a range of dilutions of rat and rabbit haemolysates.

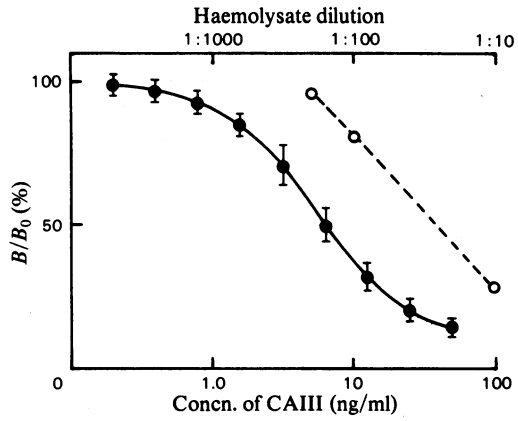

Fig. 1. *Comparison of typical standard curve for purified rat CAIII* (●) *and curve for rat haemolysate dilutions* (○)
B is the ^{125}I-labelled CAIII (c.p.m.) bound to antiserum, and B_0 is the ^{125}I-labelled CAIII (c.p.m.) bound to antiserum in the absence of unlabelled CAIII. Before the results were expressed as ratios of B/B_0, buffer non-specific binding values were subtracted from both standards and samples. Vertical bars represent ± S.D. for six replicate determinations.

Rat haemolysate was chosen, not only because it contained both CAI and CAII isoenzymes, which may have cross-reacted in the radioimmunoassay, but also to investigate the presence of CAIII in rat erythrocytes. Rabbit haemolysate was also checked for cross-reactivity, as an mRNA-dependent rabbit haemolysate has been employed to establish rat CAIII translation *in vitro* (Shiels *et al.*, 1982).

The resulting rat haemolysate displacement curve was then compared with the standard curve given by pure rat CAIII antigen. Fig. 1 shows that both curves are roughly parallel, suggesting that the cross-reactant in rat haemolysate had similar antigenic determinants to those of CAIII. In contrast, rabbit haemolysate (1 : 2 to 1 : 10 dilution) caused no significant displacements of radioactivity in the assay (results not shown). As CAIII was assayed directly (i.e. without prior extraction) in rat tissues that had not been perfused free of blood, it was important to take into account any cross-reaction with erythrocytes (Fig. 1). However, the high dilutions of tissues involved (i.e. 1 : 1000) would have served to dilute out any erythrocyte (and plasma) interference as well as to decrease non-specific binding effects to a minimum. In addition, the tissue dilutions used in the assay enabled CAIII values to be extrapolated from the linear part of the standard curve.

The monospecificity of the anti-CAIII serum is

Table 1. *Radioimmunoassay of CAIII in male and female rat tissues*

Results are expressed in terms of μg of CAIII/g of soluble protein, with the exception of those for plasma and urine, which are given as ng of CAIII/ml. Where no S.D. value is given, the values represent the means of pooled samples; otherwise all results are means ± S.D., $n \geq 5$; N.D., not determined.

Tissue	Concentration of CAIII (μg/g of soluble protein or *ng/ml)	
	Male	Female
Muscle		
Soleus	510000 ± 131000	540000
Anterior tibialis	37000 ± 12800	46000
Extensor digitorum longus	8600 ± 1400	9400
Cardiac	178.8 ± 67.5	N.D.
Liver	86400 ± 21600	2500 ± 1000
Kidney	810.4 ± 152.9	N.D.
Prostate	1800 ± 600	
Seminal vesicle	1600 ± 500	
Testis	117.7 ± 78.4	
Brain		
Hypothalamus	88.0 ± 42.4	N.D.
Amygdala	55.1 ± 11.1	N.D.
Cortex	11.7 ± 4.7	N.D.
Pituitary	12.4	N.D.
Plasma	95.6 ± 116.0*	38.9 ± 41.0*
Urine (24h collection)	12.7 ± 3.6*	N.D.

also supported by the fact that it precipitated a single polypeptide (M_r 28000) from the products of rat soleus mRNA translation *in vitro* (Shiels *et al.*, 1982).

Tissue distribution of CAIII

The distribution of CAIII in selected muscles and liver of male and female rats was determined by radioimmunoassay, and values are given in Table 1.

The distribution of CAIII in various other male rat tissues together with plasma and urine was also determined (Table 1).

Discussion

The differential expression of CAIII in numerous rat tissues was established by using a sensitive and specific radioimmunoassay method (see Table 1). Particularly high concentrations of CAIII were detected in the cytosols of soleus muscle (both sexes) and adult male liver, representing approx. 4% and 1% of total protein in these tissues respectively. Female liver and other skeletal muscles exhibited lower concentrations, and trace amounts, detectable only by radioimmunoassay, were found in other tissues, including heart, prostate, kidney, brain and erythrocytes.

If the CAIII concentration of total rat skeletal muscle is compared with that of mature male liver, then the latter tissue appears to contain about twice as much of the isoenzyme as the former (Carter *et al.*, 1981). However, immunoelectrophoretic assay of individually dissected rat skeletal muscles revealed that they contained significantly different amounts of CAIII (Carter *et al.*, 1982). Similarly, on the basis of the radioimmunoassay developed in the present work the concentration of CAIII in soleus muscle (red fibre) was 14-fold that in anterior tibialis (mixed fibre), 60-fold that in extensor digitorum longus (white fibre) and about 5-fold that in mature male liver. CAIII was found to account for up to 50% of the soluble protein in soleus muscle and 8–10% of the soluble protein in adult male liver. Previously Carter *et al.* (1982) have qualitatively shown that CAIII represents the predominant component of rat soleus soluble proteins separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Whereas no sex difference in CAIII concentration was observed between homologous muscles, the liver of female rats contained about 20–30-fold less CAIII than did liver of male rats. This sex difference with respect to liver CAIII concentration appears to be common to genetic strains of rat other than Wistar, e.g. Sprague–Dawley and Agus (A. Shiels, S. Jeffery, C. Wilson & N. Carter, unpublished work). Although there appeared to be a sex difference in plasma CAIII concentrations, the variation in values between individual rats was very large. This may partly be explained by various degrees of contamination with ruptured muscle during cardiac puncture or venepuncture. The CAIII concentrations in both plasma and urine were very low (see Table 1), indicating that the

native protein is unlikely to be actively secreted by the liver.

Relatively little is known about the true physiological function of CAIII in mammalian skeletal muscle or indeed rat liver. The fact that CAIII appears to have been highly conserved throughout evolution (Tashian *et al.*, 1983) suggests that it plays a fundamental physiological role. Sanyal *et al.* (1982) have suggested that, although CAIII displays relatively poor CO_2 hydrase activity, it occurs in high concentration, particularly in red muscle, and thus may still function in a respiratory-pH-regulatory role similar to that of the high-specific-activity CAII isoenzyme in erythrocytes. It is likely, however, that the CAII isoenzyme in liver (Carter *et al.*, 1981) would adequately perform CO_2 hydrase requirements in these tissues. In addition, the relevance of the lower but unique *p*-nitrophenyl phosphatase activity of CAIII (Koester *et al.*, 1981) to muscle physiology is unclear. The possibility that CAIII performs different functions in muscle and liver cannot be overruled. Carter *et al.* (1983) have shown that carbamoyl phosphate is a potent inhibitor of CAIII (as well as of CAI and CAII). Such inhibition may be physiologically significant, as carbamoyl phosphate is a precursor in pyrimidine synthesis and is also found in the mitochondria of urea-synthesizing tissues such as liver.

With the recent characterization of a fourth, membrane-bound, isoenzyme of carbonic anhydrase from kidney (Sanyal *et al.*, 1981) and lung Whitney & Briggle, 1982), it appears that CAIII is a member of a multigene family that provides an excellent system for investigating gene expression in various mammalian tissues.

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