Fractionation and quantification of calcium-dependent proteinase activity from small tissue samples

Abbot F. CLARK,* George N. DeMARTINO and Dorothy E. CROALL

Department of Physiology, University of Texas Health Science Center at Dallas, 5323 Harry Hines Boulevard, Dallas, TX 75235, U.S.A.

Almost all mammalian tissues contain a cytoplasmic Ca^{2+} -dependent proteolytic system consisting of two different proteinases (CDP I and CDP II) and an endogenous inhibitor specific for these proteinases. It was difficult to determine the relative activities of CDP I and CDP II directly and accurately without extensive purification, requiring relatively large amounts of tissue. We developed a simple technique based on Reactive Red–agarose affinity chromatography for quantitatively measuring CDP II activities in small (< 100 mg) tissue samples. This technique is rapid, sensitive and highly reproducible. CDP II activities can be quantified in numerous tissue samples in a single day. Using this method, we analysed the components of the CDP system in various rat tissues and demonstrated quantitative differences in CDP II activities as well as relative differences in the amounts of CDP-inhibitor activity among the various tissues. This technique should prove useful in the efforts to define the currently unknown physiological function(s) of the Ca^{2+} -dependent proteolytic system by allowing comparison of CDP activities in tissues under diverse conditions of protein metabolism.

INTRODUCTION

Ca^{2+} -dependent proteinases can be isolated from the cytosolic fraction of most mammalian cells. There are at least three distinct components of the Ca^{2+} -dependent proteolytic system: two separate Ca^{2+} -dependent proteinases (CDP I and CDP II) and an inhibitor specific for these proteinases (Murachi, 1983). CDP I and CDP II differ in their Ca^{2+} requirements for half-maximal activity: the former requires only 1–30 μM - Ca^{2+} , but the latter requires 250–500 μM - Ca^{2+} (Mellgren, 1980; Dayton *et al.*, 1981; Hathaway *et al.*, 1982; Murachi, 1983; Croall & DeMartino, 1984). CDP I and CDP II are both heterodimers, with subunits of approx. M_r 80000 and 30000. The large catalytic subunits differ in primary structure, whereas the small subunits of these proteinases are probably identical (Wheelock, 1982; Croall & DeMartino, 1984; Yumoto *et al.*, 1984).

The ubiquitous distribution of the CDP system in mammalian tissues suggests that it has an important physiological function(s). Both proteinases will endo-proteolytically cleave a variety of protein substrates (e.g. membrane proteins, membrane skeletal proteins, intermediate filaments, microfilaments, myofibrils, protein kinase C, neuropeptides etc.); however, their physiological function(s) *in vivo* is currently unknown. Several research groups have attempted to measure tissue CDP activities during altered conditions of protein metabolism (Dayton *et al.*, 1979; Brooks *et al.*, 1983; Elce *et al.*, 1984) to gain an understanding of their physiological role. However, it is difficult to measure Ca^{2+} -dependent proteolytic activity in tissue extracts quantitatively, because the CDP inhibitor is also cytoplasmic and can specifically interact

with either CDP I or CDP II (Murachi, 1983; DeMartino & Croall, 1984). In most tissues the inhibitor is present in excess relative to the proteinases and totally abrogates CDP activity in whole tissue homogenates. Therefore, in order to assess CDP activity in tissue samples, it has been necessary to fractionate tissue extracts by $(\text{NH}_4)_2\text{SO}_4$ precipitation (Dayton *et al.*, 1979; Brooks *et al.*, 1983) or gel filtration (Murachi *et al.*, 1981). Because these techniques require a relatively large amount of tissue (5–20 g), it has not been possible to quantify CDP activity in many experimental models which are amenable to the study of protein metabolism (e.g. 2–100 mg tissue samples). Immunoassays have also been used to measure Ca^{2+} -dependent proteinases (Elce *et al.*, 1983, 1984). Although these immunoassays require only small amounts of tissue, they do not directly measure CDP activity and may not allow discrimination between CDP I and CDP II.

We have developed a simple sensitive technique for quantifying CDP II activity in extracts of relatively small (100 mg) tissue samples. The endogenous CDP inhibitor and both CDP activities are separated by a single chromatography step. This technique will be useful for determining CDP activity in tissues under a variety of physiological states to assess correlations between CDP activity and specific changes in protein metabolism.

EXPERIMENTAL

Reactive Red 120–agarose was purchased from Sigma. Unsatisfactory results are obtained if it is used directly as supplied by the manufacturer. Before use, the resin is

Abbreviations used: CDP I, Ca^{2+} -dependent proteinase I (low- Ca^{2+} -requiring); CDP II, Ca^{2+} -dependent proteinase II (high- Ca^{2+} -requiring).

* To whom all correspondence should be addressed. Present address: Division of Immunology/Inflammation, Alcon Laboratories, 6201 South Freeway, Fort Worth, TX 76134, U.S.A.

washed on a sintered-glass funnel with 20 vol. (per vol. of packed resin) each of the following: distilled water, 4 M-urea, distilled water, 4 M-NaCl and distilled water. The resin is finally equilibrated and stored at 4 °C in buffer A (20 mM-Mops, 0.5 M-NaCl, 1 mM-EDTA, 1 mM-EGTA, 5 mM-2-mercaptoethanol, pH 7.5) and 0.02% Na₂S₂O₃. The resin may be reutilized many times provided that it is washed as described above before each use.

CDP I and CDP II were purified from bovine heart as reported previously (Croall & DeMartino, 1984). Purification involved chromatography on DEAE-cellulose, Sephacryl S-300, phenyl-Sepharose and Reactive Red-agarose. The CDP inhibitor was also purified from bovine heart extracts essentially as described for rat liver (DeMartino & Croall, 1984) by using Sephacryl S-300, DEAE-cellulose and CDP II-Affi-Gel 15 affinity chromatography.

Ca²⁺-dependent proteolytic activity was measured in assay buffer (35 mM-Tris/HCl, 1 mM-dithiothreitol, 7 mM-CaCl₂, pH 7.5) at 25 °C as described previously (Croall & DeMartino, 1984; DeMartino & Goldberg, 1979) by using the hydrolysis of [*methyl*-¹⁴C] α -casein to trichloroacetic acid-soluble peptides. α -Casein was reductively methylated with [¹⁴C]formaldehyde by the procedure of Dottavio-Martin & Ravel (1978). The specific activities of CDP I and CDP II were 1800–2400 and 3000–4000 caseinolytic c.p.m./min per μ g of protein respectively. CDP-inhibitor activity was measured as percentage inhibition of purified CDP II activity (0.1 μ g). The reproducibility of the caseinolytic assay is $\pm 5\%$. CDP activity in homogenate supernatants is expressed as trichloroacetic acid-soluble caseinolytic c.p.m./min per mg of homogenate supernatant protein. Tissue CDP activity is expressed as Ca²⁺-dependent caseinolytic activity with Ca²⁺-independent activity subtracted. Tissue CDP II activity which has been separated on Reactive Red-agarose is expressed as trichloroacetic acid-soluble caseinolytic c.p.m./min per mg of protein. The quantity of protein represents the amount of homogenate supernatant protein applied to the Reactive Red-agarose column. Protein determinations were by the method of Lowry *et al.* (1951), with bovine serum albumin as protein standard.

All procedures except assays were done at 0–4 °C. To determine CDP activity in tissues, approx. 100–200 mg of fresh tissue was minced and placed into 3.0 ml of buffer B (20 mM-Mops, 1 mM-EDTA, 1 mM-EGTA, 5 mM-2-mercaptoethanol, pH 7.5). The tissues were homogenized on ice with two 10 s bursts of a Brinkman Polytron at 60% maximal speed. Homogenate supernatants were prepared by centrifugation at 20000 *g* for 30 min at 4 °C. Centrifugation at 100000 *g* was not found to be necessary, since results were quite similar to those with 20000 *g* supernatants. Small plastic disposable chromatography columns (0.8 cm $\times$ 8 cm) were prepared with 2.0 ml bed volumes of Reactive Red-agarose. We generally use 1.0 ml bed volume/100 mg of tissue. This amount of resin can completely bind approx. 25 μ g of purified CDP II and will totally bind CDP II in a crude homogenate fraction from 100 mg of tissue (results not shown). The columns were equilibrated by washing with 5 vol. (per bed volume) of buffer A. Before loading on columns, the homogenate supernatants were made to 0.5 M-NaCl by the addition of 0.2 vol. of 2.5 M-NaCl in buffer B. The samples were loaded on columns and

washed with 10 bed vol. of buffer A. CDP II activity was eluted with 3 bed vol. of buffer B.

RESULTS AND DISCUSSION

Several laboratories routinely use Reactive Red-agarose affinity chromatography for the final stages of large-scale CDP II purification (Croall & DeMartino, 1984; Hathaway *et al.*, 1982). From our experience with both CDP I and CDP II on Reactive Red-agarose (Croall & DeMartino, 1984), it seemed possible that the three components of the CDP system could be fractionated by using this resin. A single chromatography step able to fractionate the proteolytic system would have significant potential for quantifying CDP activities in small tissue samples, and allow subsequent determinations of CDP activity in tissues under various physiological conditions. To test the feasibility of this approach, we have individually chromatographed pure CDP I, CDP II and CDP inhibitor on Reactive Red-agarose. These three components of the CDP system are well separated by this technique (Fig. 1). In the presence of 0.5 M-NaCl, the CDP inhibitor does not bind to the affinity matrix and is collected in a single peak. CDP I is partially retained, and is eluted as a broad peak when the Reactive Red-agarose column is further washed with buffer A (containing 0.5 M-NaCl). Recovery of most of the CDP I activity requires elution with 10 bed vol. of this buffer. CDP II is retained by the Reactive Red-agarose and is eluted as a sharp peak by changing to the buffer containing no NaCl (buffer B). Recovery of CDP II activity from the Reactive Red-agarose columns was 75–95%, depending on the amount of sample applied to the columns. For the chromatography of small (micro-

Fig. 1. Reactive Red-agarose chromatography of purified CDP I, CDP II and CDP inhibitor

Purified CDP inhibitor (▲), CDP I (○) and CDP II (●) were individually chromatographed on Reactive Red-agarose (2 ml bed volume). Fractions 1–24 were eluted with buffer A (20 mM-Mops/0.5 M-NaCl/1 mM-EDTA/1 mM-EGTA/5 mM-2-mercaptoethanol, pH 7.5). Fractions 25–49 were eluted with buffer B (buffer A without NaCl); arrow indicates change of buffer. CDP activity (trichloroacetic acid-soluble caseinolytic c.p.m./min per 100 μ l) and CDP inhibitor activity (% inhibition of CDP II activity/90 μ l) were assayed as described in the Experimental section.

gram) quantities of purified components (Fig. 1), bovine serum albumin (1 mg/ml) was included in the samples to prevent non-specific adsorption. Thus chromatography of the purified components suggested that this technique would be applicable to the fractionation of tissue extracts.

Homogenate supernatants of approx. 100 mg samples of various rat tissues were prepared and chromatographed on Reactive Red-agarose. Even in these crude homogenate supernatants, the components of the CDP system chromatographed as they did in their purified forms. To confirm the identity of the separated proteinases from homogenate supernatant as CDP I and CDP II, the Ca^{2+} -concentration requirement for caseinolytic activity was tested for each fraction. Activity in the no-salt eluate (buffer B elution) could only be detected above 0.2 mM free Ca^{2+} , confirming that only CDP II activity was being assayed. CDP activity detected in the high-salt wash required only micromolar Ca^{2+} for proteinase activity. Thus the separated proteinases had catalytic characteristics consistent with their identity as CDP I and CDP II. In addition, each enzyme reacted uniquely with antibodies specific for each proteinase (Croall & DeMartino, 1984) (results not shown).

Thus chromatography on Reactive Red-agarose separates CDP I, CDP II and CDP inhibitor from a crude supernatant in a single step. These three components of the CDP system are commonly purified by sequential steps of conventional chromatography. Gel filtration separates the CDP inhibitor from the two CDPs, whereas

DEAE-cellulose ion-exchange chromatography separates CDP II activity from CDP I and CDP inhibitor (the last two are co-eluted). However, Reactive Red-agarose chromatography offers significant advantages over gel filtration and/or DEAE-cellulose chromatography for CDP sample preparation of multiple small tissue samples. (1) Neither gel filtration nor DEAE-cellulose chromatography resolves all three CDP components. (2) Reactive Red-agarose chromatography does not require careful collection of multiple fractions from multiple columns. (3) Reactive Red-agarose chromatography does not require a salt gradient or multiple salt gradients for elution. (4) The CDP inhibitor and CDP II activity are each eluted as a sharp peak and are easy to collect, assay, and quantify.

We have subsequently used this technique to quantify CDP II activity from the homogenate supernatants of various rat tissues to establish the sensitivity and reproducibility of the method. We have quantified only CDP II activity from homogenate supernatants, although CDP I and CDP-inhibitor data could also be obtained. CDP I activity is eluted as a partially retained broad peak, and, for small tissue samples, this activity could be concentrated before assay for quantification of CDP I activity. The use of centrifuge microfiltration allows sample concentration and removal of high salt, which partially inhibits CDP I activity (results not shown).

The assay system is very sensitive. We generally use 100 mg of tissue, although the CDP II activities of single

Fig. 2. Ca^{2+} -dependent proteinase activity in rat tissues

Ca^{2+} -dependent proteinase activity was assayed in homogenate supernatants from various rat tissues: ■, activities in total homogenate supernatant; □, CDP II activities of Reactive Red-agarose-eluted homogenate supernatants. Procedures for assaying CDP activity (trichloroacetic acid-soluble caseinolytic c.p.m./min per mg of protein) and CDP II fractionation are described in the Experimental section. Abbreviation: EDL, extensor digitorum longus.

foetal mouse hearts (2–3 mg) have been determined by this technique (results not shown). This technique is also highly reproducible. Inter-assay variability was tested by determining the CDP II activities in soleus skeletal muscles from six individual rats and was $\pm 12\%$ (1807 ± 87 caseinolytic c.p.m./min per mg of protein). Intra-assay variability was tested by using six different determinations of pooled rat extensor digitorum longus skeletal-muscle homogenate supernatant and was $\pm 9\%$ (561 ± 20 caseinolytic c.p.m./min per mg of protein). Thus this technique should be able to detect changes in activity greater than 10–15% and will be useful for determining physiological and pathological variation of CDP activity in tissues.

We have determined the distribution of CDP activity in various tissues of the rat by using this Reactive Red-agarose chromatography fractionation technique (Fig. 2). The Ca^{2+} -dependent caseinolytic activity in homogenate supernatants reflects the combined activities of CDP I, CDP II and the CDP inhibitor. Homogenate supernatant CDP activity can indirectly measure the relative amount of CDP-inhibitor activity present in various tissues. CDP activity in the homogenate supernatant of many tissues is masked by the presence of excess endogenous inhibitor (Fig. 2; Murachi, 1983; Murachi *et al.*, 1981). However, three tissues (soleus skeletal muscle, lung and small intestine) have considerable CDP activity in homogenate supernatants (Fig. 2; Rodemann *et al.*, 1982). Tissue CDP II activities quantified by Reactive Red-agarose chromatography demonstrated considerable differences among the various tissues tested (Fig. 2), and these results in general agree with the work of others (Murachi *et al.*, 1981; Elce *et al.*, 1984). The considerable differences in CDP II activities between red slow-twitch oxidative (i.e. soleus) and white fast-twitch glycolytic (i.e. extensor digitorum longus) skeletal muscles may be related to the fact that the rates of protein degradation are higher in red skeletal muscle than in white skeletal muscle.

The procedure described here should allow simple and sensitive quantification of CDP II activity from relatively small amounts of tissue. CDP activities from numerous

tissue samples can be determined in a single day. This technique for the isolation and assay of CDP II activities shows potential for further defining the physiological role of the Ca^{2+} -dependent proteolytic system.

This work was supported by grants from the American Heart Association, Texas Affiliate (to A. F. C. and D. E. C.) and NIH (AM 29829) to G. N. D. A. F. C. is a postdoctoral fellow of the Arthritis Foundation. A portion of this work was presented in abstract form (Clark *et al.*, 1984).

REFERENCES

- Brooks, B. A., Goll, D. E., Peng, Y.-S., Greweling, J. A. & Hennecke, G. (1983) *Am. J. Physiol.* **244**, C175–C181
- Clark, A. F., DeMartino, G. N. & Croall, D. E. (1984) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43**, 1855
- Croall, D. E. & DeMartino, G. N. (1984) *Biochim. Biophys. Acta* **788**, 348–355
- Dayton, W. R., Schollmeyer, J. V., Chan, A. C. & Allen, C. E. (1979) *Biochim. Biophys. Acta* **584**, 216–230
- Dayton, W. R., Schollmeyer, J. V., Lepley, R. A. & Cortes, L. R. (1981) *Biochim. Biophys. Acta* **659**, 48–61
- DeMartino, G. N. & Croall, D. E. (1984) *Arch. Biochem. Biophys.* **232**, 713–720
- DeMartino, G. N. & Goldberg, A. L. (1979) *J. Biol. Chem.* **254**, 3712–3715
- Dottavio-Martin, D. & Ravel, J. M. (1978) *Anal. Biochem.* **87**, 562–565
- Elce, J. S., Hasspieler, R. & Boegman, R. J. (1983) *Exp. Neurol.* **81**, 320–329
- Elce, J. S., Baenziger, J. E. & Young, D. C. R. (1984) *Biochem. J.* **220**, 507–512
- Hathaway, D. R., Werth, D. K. & Haeberle, J. R. (1982) *J. Biol. Chem.* **257**, 9072–9077
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mellgren, R. L. (1980) *FEBS Lett.* **109**, 129–133
- Murachi, T. (1983) *Trends Biochem. Sci.* **8**, 167–169
- Murachi, T., Hatanaka, M., Yasumoto, Y., Nakayama, N. & Tanaka, K. (1981) *Biochem. Int.* **2**, 651–656
- Rodemann, H. P., Waxman, L. & Goldberg, A. L. (1982) *J. Biol. Chem.* **257**, 8716–8723
- Wheelock, M. J. (1982) *J. Biol. Chem.* **257**, 12471–12474
- Yumoto, N., Kikuchi, T., Sasaki, T. & Murachi, T. (1984) *J. Biochem. (Tokyo)* **96**, 1531–1537