Vol. 186, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1992 Pages 334-341

Amide Bond Cleavage Monitored Continuously Through Detection of a Dansylcadaverine Leaving Group

L. Lorand*, S. N. P. Murthy, K. N. Parameswaran, P. T. Velasco and J. Wilson

Department of Biochemistry, Molecular Biology and Cell Biology
Northwestern University, Evanston, IL 60208

Received May 18, 1992

The transglutaminase-catalyzed incorporation of the fluorescent amine, dansylcadaverine, into casein derivatives, such as N,N-dimethylcasein, is accompanied by a large increase in intensity of emission (Lorand et al., *Anal. Biochem.* **44**, 221-231, 1971). We have sought to make use of this sensitive detection device for the continuous, on-line monitoring of an amide-splitting reaction in which dansylcadaverine served as the leaving group. The transglutaminase-coupled test system comprised γ-glutamyl-dansylcadaverine as the first substrate and γ-glutamylamine cyclotransferase as the enzyme responsible for releasing dansylcadaverine from the γ-amide. At close to saturating levels of transglutaminase, the measured rate of increase of fluorescence, i.e. the steady-state rate of dansylcadaverine incorporation into N,N-dimethylcasein, showed a near-linear relationship with the concentration of γ-glutamylamine cyclotransferase present in the assay mixture. The general approach developed may be applicable to the assay of other amide cleaving enzymes.

The observation that the transglutaminase-catalyzed incorporation of dansylcadaverine into proteins, such as casein or its derivatives, is accompanied by a significant increase in the quantum yield of emission of the dansyl fluorophore (1), makes it possible to design novel methods for the continuous, on-line monitoring of amide splitting reactions in which dansylcadaverine is the leaving group. The coupled assay system should comprise (a) an appropriate synthetic substrate: **R**CONH$(CH_2)_5$NH·Dns[3], (b) an enzyme

* To whom correspondence should be addressed at Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, IL 60208-3500. Fax: 708-467-1380.

Abbreviations: Dns = dansyl; Dc = dansylcadaverine in the amide bond; Bzl = benzyl; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; γ-GACT = γ-glutamylamine cyclotransferase.

0006-291X/92 $4.00

specific for cleaving the -CONH-bond in this substrate, (c) a transglutaminase (E.C. 2.3.2.13), (d) a casein derivative, such as N,N-dimethylcasein, to act as acceptor for the transglutaminase-mediated incorporation of the released dansylcadaverine product and (e) Ca^{2+}-ions essential for activating transglutaminase. The feasibility of this analytical approach was tested with the use of γ-glutamyldansylcadaverine (γ-Glu-Dc) as the first substrate and γ-glutamylamine cyclotransferase (2) as the catalyst for releasing dansylcadaverine from this γ-amide.

Materials and Methods

Synthesis of N^1-L-(γ-glutamyl)-N^5-[5-dimethylamino)-1-naphthalenesulfonyl]diaminopentane (γ-glutamyldansylcadaverine; compound I). This was synthesized according to a published procedure (3).

Synthesis of N^1-[γ-(N^α-Boc)glutamyl]-N^5-[5-dimethylamino]-1-naphthalenesulfonyl]diaminopentane (compound II). The completely blocked intermediate, N^α-Boc-α-benzyl-γ-glutamyldansylcadaverine, was partially deblocked by catalytic hydrogenation as described (3) to give compound II.

Synthesis of N^1-L-[γ-(α-benzyl)glutamyl]-N^5-[5-dimethylamino)-1-naphthalenesulfonyl]diaminopentane (compound III). The blocked intermediate, N^α-Boc-α-benzyl-γ-glutamyldansylcadaverine (3), was partially deblocked with 50% trifluoroacetic acid in dichloromethane as described under the synthesis of compound I (3) to give the trifluoroacetate salt of III, homogeneous by TLC (fluorescent and ninhydrin positive); R_f 0.74 (chloroform/methanol/acetic acid 10:2:1), 0.73 (n-butanol/acetic acid/water 15:6:5), 0.1 (chloroform/acetic acid 10:1), and 0.82 (1-propanol/water/NH_4OH/ethanol/ethyl acetate 7:4:2:3:10).

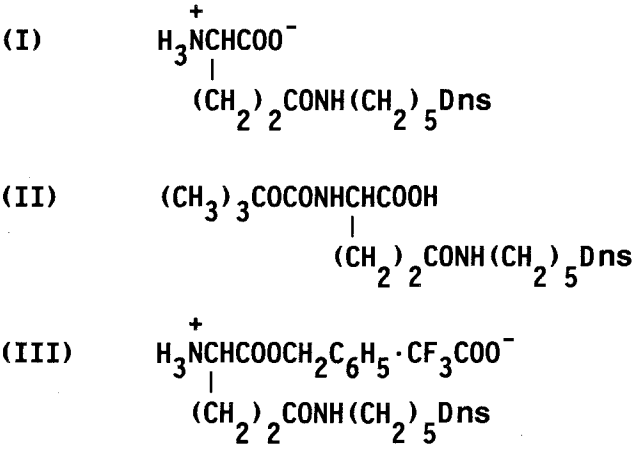
γ-Glutamylamine cyclotransferase-catalyzed release of dansylcadaverine. Reactions were carried in mixtures of 40 μl comprising 50 mM Tris-HCl, pH 7.5, 10% (v/v) ethanol, 0.2 mM of one of the fluorescent compounds, and 0-20 μg/ml of γ-glutamylamine cyclotransferase (2). Following incubation at 37° C for 30 min, 2 μl aliquots were spotted on a TLC plate (Polygram 0.1 mm Polyamide-6; Macherey & Nagel, Alltech Associates, Deerfield, IL) and the plate was developed for 60 min in aqueous 1% pyridine (pH 5.4) (4). The dried plates were photographed under uv light (366 nm).

Coupled enzyme assay. N,N-dimethylcasein was prepared from Hammersten casein (United States Biochemical Corp., Cleveland, OH) using the procedure of Lin et al. (5) and its concentration (in 1 ml of 50 mM Tris-HCl, pH 7.5) was determined using the BCA protein assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions with bovine serum albumin as standard. γ-Glutamylamine cyclotransferase was purified from rabbit kidney according to Fink et al. (2). Activity of the enzyme (3.6 μg) was tested in reaction with γ-GluDc (100 nmoles of substrate in 100 μl of 50 mM sodium phosphate, pH 7.5, 30 min at 37° C) by measuring the release of free dansylcadaverine, as described by Cariello et al. (3). During this period, 73% of the amide was cleaved, giving an apparent specific activity of 40 μmoles of product/hr per mg of enzyme, which is seemingly higher than the literature report (2). Guinea pig liver transglutaminase was purified by David Schilling to apparent SDS-PAGE homogeneity based on a published procedure (6). The concentration of transglutaminase was obtained by measuring its absorbancy at 280 nm in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA (7). Its activity was measured, according to the procedure of Lorand et al. (8), by the incorporation of ^{14}C-putrescine (0.5 mM at 10 mCi/mmole) into N,N-dimethylcasein (5 mg/ml) in a 20 min reaction at pH 7.5 in 50 mM Tris-HCl, as 186 μmoles of ^{14}C-putrescine/hr per mg of enzyme when measured with 5 mg/ml N,N-dimethylcasein.

The coupled enzyme assay was typically performed at 37° C in a reaction mixture of about 0.5 ml containing 1.0 mg/ml dimethylcasein, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1-30 μM γ-glutamyldansylcadaverine, 5 mM $CaCl_2$, 10 μg/ml transglutaminase and 2 μg/ml γ-glutamylamine cyclotransferase being added last. In controls either γ-glutamylamine cyclotransferase or transglutaminase were omitted from the reaction mixture. In EDTA controls 5 mM EDTA replace $CaCl_2$. The increase in the relative fluorescence intensities (λ_{exc} = 330 nm; λ_{em} = 500 nm) was recorded using an Aminco-Bowman spectrophotofluorometer.

Results and Discussion

Three synthetic compounds (I-III), all γ-amide derivatives of glutamic acid and containing dansylcadaverine as potential leaving group, were tested for substrate activity with γ-glutamylamine cyclotransferase.



The γ-glutamylamine cyclotransferase-catalyzed release of free dansylcadaverine was examined with the use of a published thin-layer chromatographic procedure (4), as illustrated in Fig. 1. It is seen that, of the three substances, only compound I (i.e. γ-Glu-Dc) proved to be a substrate for γ-glutamylamine cyclotransferase yielding free dansylcadaverine upon incubation with this enzyme. As shown in lane 3, nearly all the amide (8 nmoles) was cleaved by the enzyme (0.4 μg) in 30 min, which translates to an activity of close to 40 μmoles of dansylcadaverine released/hr per mg enzyme. Thus, further studies for constructing the continuous, on-line kinetic assay system made use of γ-Glu-Dc as the first substrate.

Typically, reaction mixtures comprised the following constituents in a total volume of 0.5 ml: γ-Glu-Dc (0-30 μM), transglutaminase (0-6.25 μg), N,N-dimethylcasein (0.5 mg), 150 mM NaCl, 50 mM Tris-HCl buffer of pH 7.5, 5 mM $CaCl_2$ and γ-glutamylamine cyclotransferase (0-1 μg). The solutions were placed in the spectrophotofluorometer (37° C) for recording changes in relative fluorescence intensities (λ_{exc} = 330 nm, λ_{em} =

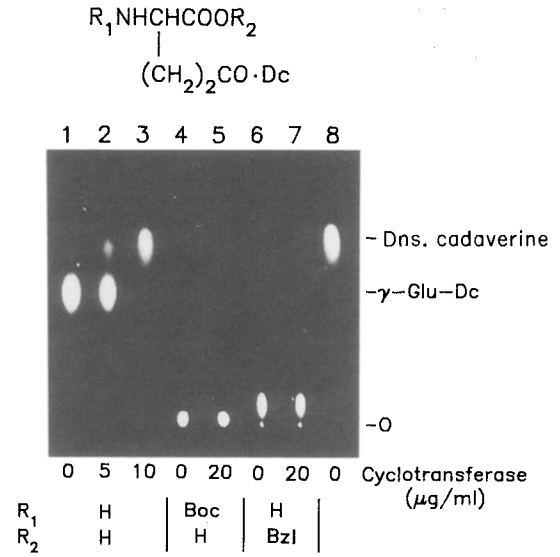


Fig. 1. Release of dansylcadaverine from γ-glutamyldansylcadaverine, as catalyzed by γ-glutamylamine cyclotransferase. γ-Glutamylamine cyclotransferase (0-20 μg/ml) was incubated with 0.2 mM γ-glutamyldansylcadaverine (lanes 1-3) or its blocked derivatives [where R_1 = *tert*-butyloxycarbonyl (lanes 4 and 5) or R_2 = benzyl (lanes 6 and 7)] at 37° C for 30 min in a buffer containing 50 mM Tris-HCl, pH 7.5 and 10% (v/v) ethanol. The samples were evaluated by TLC as described in Materials and Methods. Lane 8 contained 0.2 mM dansylcadaverine as a reference. Dc, dansylcadaverine; O, origin.

500 nm). As seen in Fig. 2, line 1, the progression curve reflecting the incorporation of the dansylcadaverine product into casein, following a brief lag phase, contained a near-linear segment from which the steady-state velocity could be calculated. If γ-glutamylamine cyclotransferase was omitted from the assay (Fig. 2, line 2), no change in fluorescence ensued, proving the validity of the coupled enzyme system for measuring γ-glutamylamine cyclotransferase activity.

It had to be demonstrated that the recorded changes in fluorescence intensities were obtained at sufficiently high concentrations of the monitoring enzyme that the activity of the latter would not be rate limiting. Indeed, the data presented in Fig. 3 clearly show that, with 2 μg/ml of γ-glutamylamine cyclotransferase in the mixture, the 10 μg/ml concentration of transglutaminase employed in Fig. 2 (line 1) represented a close to saturating level in this regard. Subsequent experiments were carried out with this concentration of the transglutaminase added.

It was desireable to express velocities for the release of dansylcadaverine in the amide-cleaving reaction, measured by the coupled assay, in terms of moles of dansylcadaverine released per unit weight of γ-glutamylamine cyclotransferase per unit

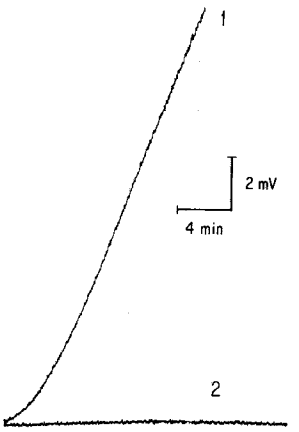


Fig. 2. Increase in fluorescence intensity measured by the coupled assay as dansylcadaverine was cleaved from γ-glutamyldansylcadaverine by the action of γ-glutamylamine cyclotransferase and the released dansylcadaverine was incorporated into N,N-dimethylcasein through the action of transglutaminase. Curve 1 represents a typical reaction containing 1 mg/ml of dimethylcasein, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 μM γ-glutamyldansylcadaverine, 5 mM $CaCl_2$, 10 μg/ml of transglutaminase and 2 μg/ml of γ-glutamylamine cylcotransferase. Curve 2 shows three controls with omissions of γ-glutamylamine cyclotransferase or transglutaminase or $CaCl_2$.

time. Therefore, ΔF/t values, given in mV/min, had to be evaluated in terms of the amount of dansylcadaverine that would be available for the transglutaminase-catalyzed trapping reaction. For this purpose, steady-state initial velocities of fluorescence

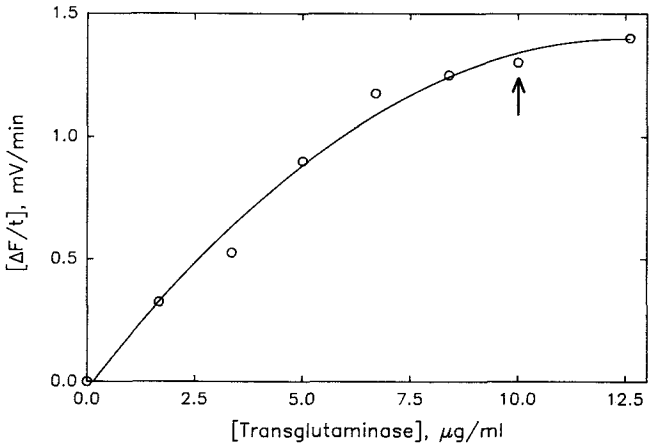


Fig. 3. Selecting a saturating level for transglutaminase, where the change in fluorescence in the coupled system (ordinate) would no longer depend on the concentration of this monitoring enzyme. The mixtures contained 20 μM γ-GluDc, 2 μg/ml of γ-glutamylamine cyclotransferase and varying concentrations (abscissa) of transglutaminase. The arrow marks the concentration of transglutaminase used in the experiment for Fig. 2 and for subsequent experiments with the coupled assay system.

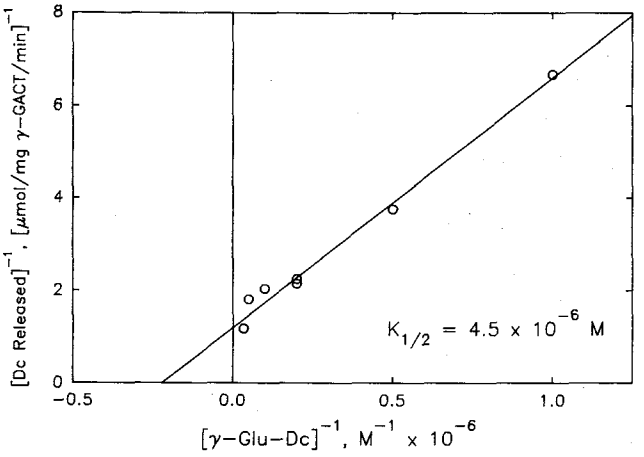


Fig. 4. Double reciprocal plot for the velocity of release of dansylcadaverine from γ-GluDc as substrate for γ-glutamylamine cyclotransferase (2 μg/ml), measured through the transglutaminase (10 μg/ml)-catalyzed incorporation of the dansylcadaverine product into N,N-dimethylcasein.

increases were measured in the direct transglutaminase (10 μg/ml)-mediated incorporation of dansylcadaverine (from 0 to 2.5 μM) into N,N-dimethylcasein (1 mg/ml) in the presence of Ca^{2+}. The calibration curve (not shown) relating ΔF/t to dansylcadaverine concentration was employed for calculating the rates of release of dansylcadaverine with the coupled assay in Figs. 4 and 5.

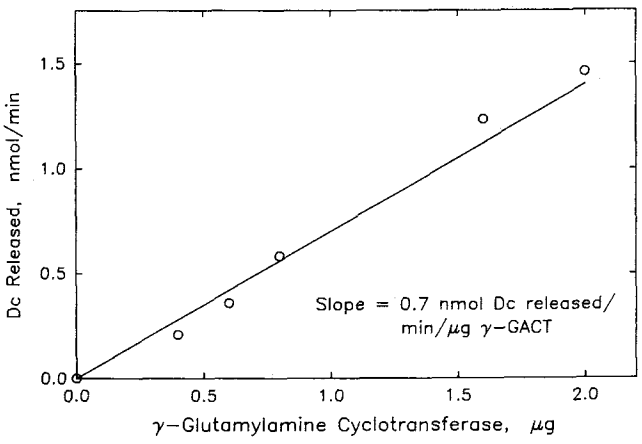


Fig. 5. Reaction velocities (ordinate) for the cleavage of γ-GluDc (20 μM) at various concentrations of γ-glutamylamine cyclotransferase (abscissa), as monitored by the transglutaminase (10 μg/ml)-catalyzed incorporation of released dansylcadaverine into N,N-dimethylcasein.

The dependence of the overall reaction velocity of the γ-glutamylamine cyclotransferase- and transglutaminase-containing coupled system on the concentration of γ-GluDc was examined as shown in Fig. 4. From the double reciprocal plot, an apparent half-saturation value of $K_{1/2}$ of about 4.5×10^{-6} M was calculated for γ-GluDc. It can thus be concluded that the experiments presented in Figs. 2 and 3 were carried out with initial concentrations of γ-GluDc which were about four-fold higher than half-saturating by this substrate. V_{max} was calculated as 0.8 μmoles of Dc released/min per mg γ-glutamylamine cyclotransferase, or 48 μmoles/hr per mg (which compares well with the specific activity reported in the Materials and Methods section).

Fig. 5 shows that, by optimizing the concentrations of γ-GluDc and transglutaminase, the coupled assay system responds in a near-linear fashion to changes in the concentration of γ-glutamylamine cyclotransferase within the illustrated range of 0-2 μg/ml of the enzyme tested. The demonstration establishes the validity of the transglutaminase-based monitoring approach for measuring the release of dansylcadaverine from its amide. From the slope of the line in Fig. 5, the specific activity of the γ-glutamylamine cyclotransferase was calculated as 42 μmoles of Dc released/hr per mg enzyme, in excellent agreement with the value derived from the V_{max} in Fig. 4.

Cleavage of the γ-amides by γ-glutamylamine cyclotransferase proceeds with formation of pyroglutamate (2). However, from the point of view of utilizing the transglutaminase-based detection of dansylcadaverine, it would not matter whether this product was generated by cyclic elimination or by hydrolysis. Thus, it may be inferred that the on-line monitoring of fluorescence, as described, would also be applicable for assaying a variety of enzymes which catalyze the hydrolytic removal of dansylcadaverine from γ-amides. Different enzymes might require synthesis of different γ-Glu-Dc derivatives as specific first substrates; for example, considering the suggested mode of action for "destabilase", an enzyme thought to promote exclusively the cleavage of protein-to-protein N^{ϵ}-(γ-glutamyl)lysyl bridges (9), use of a fully blocked derivative of γ-Glu-Dc, such as

$$CH_3CONHCHCONHCH_3$$
$$|$$
$$(CH_2)_2CONH(CH_2)_5NH\cdot Dns$$

could be suggested as possible substrate.

Enzymes which might act to remove dansylcadaverine from β-amides, such as β-Asp-Dc and its blocked derivatives, could also be studied by the transglutaminase-dependent coupled assay presented in this paper. This type of system seems to be particularly well suited for testing enzymes such as γ-glutamylamine cyclotransferase with specificity for amides of alkylamines, where no direct colorimetric or fluorometric methods

are now available for measuring the removal of the amine moiety by a continuous analytical procedure. While testing for release of dansylcadaverine from α-amide linkages might also be possible, complications could arise by attack of the peptide backbone of the casein substrate by the α-amide hydrolyzing enzymes themselves.

Acknowledgments: This work has been suppported by a USPHS Research Career Award (HL-03512) and by grants from the National Institutes of Health (EY-03942, HL-16346, and HL-45168).

References

1. Lorand, L., Lockridge, O. M., Campbell, L. K., Myhrman, R., and Bruner-Lorand, J. (1971) *Anal. Biochem.* **44**, 221-231.
2. Fink, M. L., Chung, S. I., and Folk, J. E. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4564-4568.
3. Cariello, L., Velasco, P. T., Wilson, J., Parameswaran, K. N., Karush, F., and Lorand, L. (1990) *Biochemistry* **29**, 5103-5108.
4. Lorand, L., and Campbell, L. K. (1971) *Anal. Biochem.* 44, 207-220.
5. Lin, Y., Mean, G. E., and Feeney, R. E. (1969) *J. Biol. Chem.* **244**, 789-793.
6. Croall, D. E., and DeMartino, G. N. (1986) *Cell Calcium* **7**, 29-39.
7. Folk, J. E., and Cole, P. W. (1966) *J. Biol. Chem.* **241**, 5518-5525.
8. Lorand, L., Campbell-Wilkes, L. K., and Cooperstein, L. (1972) *Anal. Biochem.* **50**, 623-631.
9. Baskova, I. P., and Nikonov, G. I. (1991) *Blood Coagulation and Fibrinolysis* **2**, 167-172.