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Purification of a Novel Calmodulin Binding Protein from Bovine Cerebral Cortex Membranes†

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ABSTRACT: A new calmodulin (CaM) binding protein, designated P-57, has been purified to apparent homogeneity from bovine cerebral cortex membranes. In contrast to other calmodulin binding proteins, P-57 has higher affinity for calmodulin in the absence of bound Ca^{2+} than in its presence. The protein was purified by DEAE-Sephacel chromatography and two CaM–Sepharose affinity column steps. The first CaM–Sepharose column was run in the presence of Ca^{2+}; the second was run in the presence of chelator in excess of Ca^{2+}. P-57 was adsorbed by CaM–Sepharose only in the absence of bound Ca^{2+} and was eluted from the second column by buffers containing Ca^{2+}. Sodium dodecyl sulfate (SDS)–polyacrylamide gels of the purified protein showed only one band at M_r 57 000. The major form of the protein on Bio-Gel A-1.5m and native polyacrylamide gradient gel electrophoresis ran with an apparent Stokes radius of 41 Å. Photoaffinity labeling of P-57 with azido[^{125}I]calmodulin yielded one cross-linked product on SDS gels with an M_r of 70 000. This interaction occurred only when excess ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid was present and was inhibited by the presence of Ca^{2+} in excess of chelator. It appears that P-57 has novel binding properties for calmodulin distinct from all other calmodulin binding proteins described thus far.

Calmodulin (CaM)[1] functions as an important intracellular Ca^{2+} receptor and mediates Ca^{2+} stimulation of a variety of enzymes (Cheung, 1970; Wang & Waisman, 1979; Klee et al., 1980; Klee & Vanaman, 1982). Binding between CaM and CaM binding proteins has been directly demonstrated by a number of techniques including gel filtration (Teshima & Kakiuchi, 1974), electrophoresis on nondenaturing gels (Amphlett et al., 1976; LaPorte & Storm, 1978), CaM–Sepharose affinity chromatography (Watterson & Vanaman, 1976; Klee & Krinks, 1978; Wescott et al., 1979), fluorescence techniques (LaPorte et al., 1981), and cross-linking of [^{125}I]CaM to CaM binding proteins (LaPorte et al., 1979; Andreasen et al., 1981). Although Ca^{2+} binding to CaM generally enhances its affinity for CaM binding proteins, it is clear from thermodynamic considerations (Keller et al., 1980, 1982a,b) that CaM must have finite affinity for CaM binding proteins even in the absence of bound Ca^{2+}. For example, calcium-independent binding of CaM to troponin I has been directly demonstrated with fluorescent labeled proteins (Olwin et al., 1982). As of yet, however, there has been no report of any CaM binding protein whose affinity for CaM is lowered by Ca^{2+}.

In this paper, we report the discovery and purification of a membrane protein from brain that apparently has higher affinity for CaM in the absence of bound Ca^{2+} and is dissociated from CaM by Ca^{2+}. It is proposed that this protein, designated P-57, may function to localize and concentrate CaM at the membrane surface and release free CaM in response to increases in intracellular free Ca^{2+}.

Materials and Methods

Materials

DEAE-Sephacel and cyanogen bromide activated Sepharose 4B were obtained from Pharmacia. Enzymobeads and Bio-Gel A-1.5m were obtained from Amersham. All other reagents

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[1] Abbreviations: CaM, calmodulin; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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were of the finest available grade from commerical sources.

Methods

Preparation of CaM. CaM was prepared from bovine brain as described by LaPorte et al. (1979). CaM–Sepharose was prepared from purified CaM and cyanogen bromide activated Sepharose 4B according to the procedure of Westcott et al. (1979). Protein concentrations were determined by the methods of Peterson (1977).

Purification of P-57. One kilogram of bovine cerebral cortex was homogenized in an equal volume of homogenization buffer with a Waring blender for 30 s. Homogenization buffer contained 20 mM glycylglycine, pH 7.2, 1 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose, 3 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride. The preparation was then further disrupted by three strokes of a Dounce homogenizer using a loose-fitting pestle and centrifuged at 7000 rpm for 30 min in a Sorval GSA rotor. The pelleted membranes were resuspended in an equal volume of homogenization buffer and the Dounce homogenization, centrifugation, and resuspension were repeated three times. The final pellet was resuspended in 20 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiothreitol and centrifuged as before. The washed membrane pellet was detergent extracted by addition of 20 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1 mM $MgCl_2$, 1 mM EDTA, and 1% Lubrol PX to a detergent to protein ratio of 2.5:1 (w/w). The mixture was stirred at 4 °C for 60 min and centrifuged at 10000*g* for 2 h, and the supernatant fluid was decanted. Two liters of DEAE-Sephacel equilibrated in 50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% Lubrol PX (buffer A) was incubated for 90 min with 5 L of detergent extract with stirring at 4 °C. The ion exchange was washed on a sintered glass funnel with 4 L of buffer A containing 50 mM KCl, poured into a 9 × 30 cm column, and eluted with buffer A containing 150 mM KCl. A single protein peak containing the P-57 was eluted. CaM, which did not elute from DEAE-Sephacel below 300 mM KCl, did not emerge. The DEAE-Sephacel pool was diluted with an equal volume of buffer A containing 2.2 mM $CaCl_2$. The dilution lowered the KCl concentration of the pooled solution from approximately 110 to 55 mM and provided Ca^{2+} in excess of EDTA. This sample was loaded onto a 2.5 × 26 cm CaM–Sepharose column equilibrated in buffer A containing 1.1 mM $CaCl_2$. The flowthrough from this first CaM–Sepharose column (F_1) contained P-57 whereas other CaM binding proteins were adsorbed. The column was washed with equilibration buffer, until the absorbance at 280 nm reached a steady value, and was eluted with buffer A. This preparation is designated F_2, and it contained the CaM-sensitive adenylate cyclase, a cyclic nucleotide phosphodiesterase, and other CaM binding proteins that have higher affinity for $CaM{\cdot}Ca^{2+}{}_n$ than CaM (Andreasen et al., 1983).

EGTA was added to F_1 to a final concentration of 5 mM, and the sample was reapplied to CaM–Sepharose and washed with 5 column volumes of buffer A containing 5 mM EGTA. P-57 was eluted from CaM–Sepharose with buffer A containing 3 mM $CaCl_2$.

Preparation and Use of Azido[^{125}I]CaM. CaM was iodinated to a specific activity of (0.5–1) × 10^8 cpm/nmol by the Enzymobead method (Bio-Rad). [^{125}I]CaM was desalted into and dialyzed against 50 mM borate (pH 9.8)–100 mM NaCl. MABI (Pierce) was added to a 20-fold excess over [^{125}I]CaM, and the mixture was incubated for 2 h in the dark at room temperature. The reaction products were dialyzed with 20 mM MOPS (pH 7.2)–100 mM NaCl in a flow dialysis cell for 2 days in the dark at 4 °C. The azidification and both dialysis steps were carried out in the dialysis cell. This afforded convenient handling and a minimum of sample transference and allowed up to 0.5 mg of azido[^{125}I]CaM to be synthesized at a time.

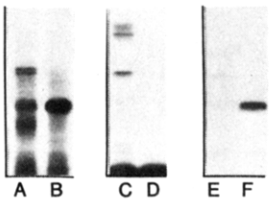

FIGURE 1: Azido[^{125}I]CaM photoaffinity labeling at various stages of purification of P-57. Samples were incubated with azido[^{125}I]CaM, run on SDS gels, and autoradiographed as described under Methods. Solubilized cerebral cortex membranes (20 μg) with (lane A) 1 mM $CaCl_2$, (lane B) 2 mM EGTA, (lane C) 10 μg of F_2 + 1 mM $CaCl_2$, (lane D) 10 μg of F_2 + 2 mM EGTA, (lane E) 20 μg of F_1 + 1 mM $CaCl_2$, and (lane F) 20 μg of F_1 + 2 mM EGTA.

Photolysis experiments were carried out as follows: Ingredients were mixed in 1.5-mL microfuge tubes under low light and transferred to a nine-well Pyrex spot plate (Corning 7220, VWR Scientific). Samples (up to six at a time) were irradiated for 2 min on ice with Mineralight UVS-11 placed directly onto the spot plate. Cross-linked products were visualized as autoradiography bands from dried SDS slab gels (Laemmli, 1970) on Du Pont Cronex Lightning Plus intensifying screens and Kodak XR-5 X-ray film.

Electrophoresis. Native polyacrylamide gradient slab gels were prepared and run as previously described (Andreasen et al., 1983). Protein standards used for estimation of Stokes radii were aldolase (47 Å), ovalbumin (27 Å), and the Pharmacia high molecular weight kit. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out by the method of Laemmli (1970). Autoradiography was carried out on dried slab gels at −80 °C with Kodak XR-5 X-ray film.

Results

Purification of P-57. This protein was originally detected in detergent-solubilized cerebral cortex membranes by virtue of its formation of a cross-linked product with azido[^{125}I]CaM in the presence of excess EGTA (Figure 1, lane B). This crude membrane preparation contained several other CaM binding proteins that all required Ca^{2+} for interaction with azido-[^{125}I]CaM (Figure 1, lane A). The lower affinity of P-57 for $CaM{\cdot}Ca^{2+}{}_n$ compared to CaM was exploited for purification of the protein. Those proteins (F_2) that have higher affinity for CaM when Ca^{2+} is present were removed by adsorption to CaM–Sepharose with excess Ca^{2+} present. F_2 was eluted from CaM–Sepharose with excess chelator, and cross-linked products were formed with azido[^{125}I]CaM only when free Ca^{2+} was present in excess over chelator (Figure 1, lanes C and D). In contrast, P-57 present in F_1 only formed a cross-linked polypeptide with azido[^{125}I]CaM when EGTA was in significant excess over Ca^{2+} (Figure 1, lanes E and F).

Purification to apparent homogeneity was accomplished by Lubrol PX solubilization from membranes, DEAE-Sephacel chromatography to remove endogeneous CaM, and two CaM–Sepharose columns (±Ca^{2+}). P-57 was adsorbed to CaM–Sepharose in the presence of excess EGTA; the column was washed extensively with chelator-containing buffers, and P-57 eluted as a single protein peak by use of 3 mM $CaCl_2$ (Figure 2). This protein peak consisted (>95%) of a single polypeptide having an M_r of 57 000 on SDS gels; overloaded gels revealed little other protein (Figure 2, insert). It is estimated that P-57 accounted for approximately 0.2% of the

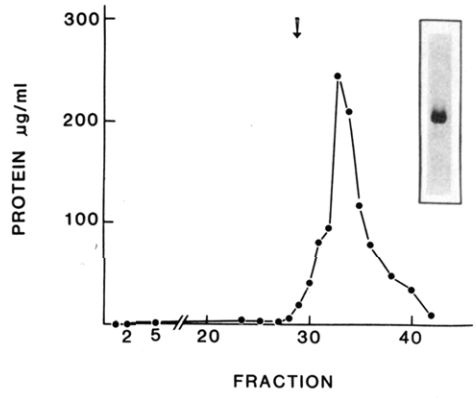

FIGURE 2: Elution of P-57 from CaM–Sepharose by Ca^{2+}. F_1 was loaded into a 130-mL CaM–Sepharose column, washed, and eluted with Ca^{2+} as described under Methods. The 3 mM Ca^{2+} elution buffer was started at fraction 1 after a five-column wash with 5 mM EGTA. P-57 eluted coincident with the Ca^{2+} front (indicated by the arrow). The insert shows an SDS gel of 15 μg of protein obtained from the protein peak.

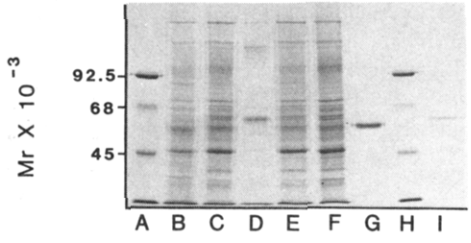

FIGURE 3: SDS gels at various stages of purification of P-57. Fractions at various stages of the purification were run on SDS gels and stained with Coomassie Brilliant Blue as described under Methods. (Lane A) Bio-Rad high molecular weight standards; (lane B) 20 μg of solubilized bovine cerebral cortex membranes; (lane C) 20 μg of pooled protein from DEAE-Sephacel; (lane D) 10 μg of F_2; (lane E) 20 μg of F_1; (lane F) 20 μg of flowthrough from the second CaM–Sepharose column; (lane G) 5 μg of purified P-57; (lane H) Bio-Rad high molecular weight standards; (lane I) 2 μg of bovine heart CaM-sensitive phosphodiesterase.

detergent-solubilized membrane preparation. Approximately 10 mg of P-57 was obtained from 1 kg of bovine cerebral cortex. It is evident from SDS gels of samples taken at various stages of the purification that most of the purification occurred on the second CaM–Sepharose column (Figure 3). It also appears that P-57 is the only protein in the membrane preparation with higher affinity for CaM in the absence of free Ca^{2+} than in the presence of Ca^{2+}.

Cross-Linking of Azido[^{125}I]CaM to Purified P-57. The purification scheme described above indicated that P-57 exhibited unusual CaM binding properties. Photoaffinity cross-linking of azido[^{125}I]CaM to purified P-57 confirmed that the protein does indeed have higher affinity for CaM than CaM·Ca^{2+}_n (Figure 4). A single cross-linked product of M_r 70000 was obtained only when EGTA was in excess over Ca^{2+}. The presence of a 15-fold excess of unmodified CaM over the azido derivative greatly reduced the amount of 70000-dalton product formed. These results suggest a 1:1 stoichiometry for binding of CaM to P-57. Although the Ca^{2+} dependency for P-57 interaction with CaM appears reversed compared to other CaM binding proteins, the binding stoichiometry is the same as for the other systems (Andreasen et al., 1981).

Native Size of P-57. Native polyacrylamide gradient gel electrophoresis of purified P-57 suggested that native P-57 may have quaternary structure with monomers associating to form larger particles (Figure 5). On a 4–20% linear gradient, P-57 appeared as a single band by protein staining. The P-57 peak migrated with the same mobility as lactate dehydrogenase

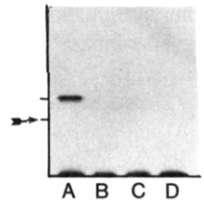

FIGURE 4: Photoaffinity labeling of purified P-57 with azido[^{125}I]CaM. P-57 (1 μg) was photoaffinity labeled with azido[^{125}I]CaM, subjected to electrophoresis in SDS, and autoradiographed as described under Methods. (Lane A) Photolysis carried out in 3 mM EGTA; (lane B) same as (A) but with 5-min preincubation of P-57 with 15 μM unmodified CaM; (lane C) photolysis carried out in 3 mM $CaCl_2$; (lane D) same as (C) but with preincubation with 15 μM unmodified CaM. Unmodified P-57, located by protein stain, ran at the arrow.

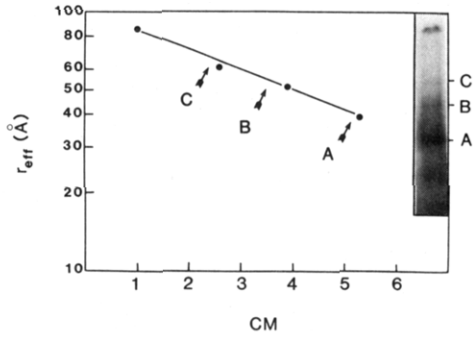

FIGURE 5: Native gradient gel electrophoresis of P-57. P-57, which had been cross-linked to azido[^{125}I]CaM, was applied to a linear 4–20% polyacrylamide gradient run at 150 V for 35 h and autoradiographed as indicated under Methods. Calibration standards included thyroglobulin (Stokes radius, 85 Å), ferritin (60 Å), catalase (52 Å), and lactate dehydrogenase (40 Å). (Left panel) Autoradiogram showing major (A) and minor (B, C) cross-linked products; (insert) location of A, B, and C on the autoradiogram.

(Stokes radius, 40 Å). Autoradiography of a parallel gel lane loaded with P-57 cross-linked to azido[^{125}I]CaM revealed a major product a little larger than the lactate dehydrogenase standard (Figure 5). Prolonged autoradiography of this gel revealed several bands at higher Stokes radii (54 and 65 Å). When these bands were excised and run in SDS in a second dimension, they all gave a single product of M_r 70000. It appears that each subunit can bind one CaM and that the system may associate to form multimeric particles.

Discussion

There are significant amounts of CaM associated with crude membrane preparations from bovine cerebral cortex, and it has been our general observation that it is difficult to remove all of the CaM, even with extensive washing with chelator-containing buffers. The presence of relatively large amounts of P-57 (0.2% of membrane protein) in the membranes may explain, at least in part, these observations. P-57 was purified to apparent homogeneity by DEAE-Sephacel and two CaM–Sepharose columns. Its unusual affinity for CaM in the presence of excess chelator allowed the protein to be purified relatively easily. In fact, the purification on the second CaM–Sepharose column is quite striking compared to that normally achieved with other CaM binding proteins using CaM–Sepharose.

The behavior of P-57 on CaM–Sepharose and cross-linking studies with azido[^{125}I]CaM clearly illustrate the preferential affinity of the protein for CaM in the absence of bound Ca^{2+}. Although dissociation constants for P-57 and CaM have not yet been rigorously quantitated, their affinity for CaM–Sepharose with excess chelator and preliminary fluorescence studies suggest a K_D in the micromolar range. Binding between CaM and CaM binding proteins without Ca^{2+} has been

reported for several other systems including troponin I (Olwin et al., 1982), phosphorylase kinase (Cohen et al., 1978), and *Bordetella pertussis* adenylase cyclase (Greenlee et al., 1982; Kilhoffer et al., 1983). However, all of these proteins exhibit higher affinity for CaM·Ca^{2+}_n than CaM. P-57 is the first CaM binding protein discovered that actually has higher affinity for CaM compared to CaM·Ca^{2+}_n.

We have avoided the temptation of naming P-57 more specifically until its function and relationship to other CaM binding proteins are more thoroughly examined. Thus far, we have been unable to detect kinase, phosphodiesterase, phosphatase, adenosinetriphosphatase, or adenylate cyclase activity associated with the pure protein. It is possible that P-57 is a proteolytic fragment derived from some other CaM binding protein; however, its unusual CaM binding properties make that possibility seem unlikely. The most interesting property of P-57 is its relative affinities for CaM in the presence and absence of Ca^{2+}, which suggest one possible function of P-57. This protein subunit may function to bind CaM at some local site (e.g., a multisubunit enzyme complex or the inner surface of the membrane), thereby concentrating and localizing CaM in the cell. Increases in free Ca^{2+} in response to a stimulus would result in the release of CaM from P-57. Such stimulus-induced release of CaM from membrane sites has been detected in rat brain (Gnegy et al., 1977). For example, a protein such as P-57 could be used to localize CaM near Ca^{2+} channels. The relatively large amounts of P-57 present in cerebral cortex do suggest that a significant amount of the total CaM present will be associated with P-57 when Ca^{2+} levels are low (<0.1 μM). P-57 was not detected in a variety of other tissues including bovine heart, rat heart, liver, lung, and skeletal muscle (data not shown). Definition of the function of P-57 in brain will clearly require further experimentation.

Registry No. Calcium, 7440-70-2.

References

Amphlett, G. W., Vanaman, T. C., & Perry, S. V. (1976) *FEBS Lett. 72*, 163–168.

Andreasen, T. J., Keller, C. H., LaPorte, D. C., Edelman, A. M., & Storm, D. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2782–2785.

Andreasen, T. J., Heideman W., Rosenberg, G. B., & Storm, D. R. (1983) *Biochemistry 22*, 2757–2762.

Cheung, W. Y. (1970) *Biochem. Biophys. Res. Commun. 38*, 533–538.

Cohen, P., Burchell, A., Foulkes, J. G., Cohen, P. T. W., Vanaman, T. C., & Nairn, A. (1978) *FEBS Lett. 92*, 287.

Gnegy, M. E., Nathanson, J. A., & Uzunov, P. (1977) *Biochim. Biophys. Acta 497*, 75–85.

Greenlee, D. V., Andreasen, T. J., & Storm, D. R. (1982) *Biochemistry 21*, 2759.

Keller, C., LaPorte, D. C., Toscano, W. A., Storm, D. R., & Westcott, K. R. (1980) *Ann. N.Y. Acad. Sci. 356*, 205–219.

Keller, C. H., Olwin, B. B., LaPorte, D. C., & Storm, D. R. (1982a) *Biochemistry 21*, 156–162.

Keller, C. H., Olwin, B. B., Heideman, W., & Storm, D. R. (1982b) *Calcium Cell Funct. 3*, 103–127.

Kilhoffer, M.-C., Cook, G. H., & Wolff, J. (1983) *Eur. J. Biochem. 133*, 11–15.

Klee, C. B., & Krinks, M. H. (1978) *Biochemistry 17*, 120–126.

Klee, C. B., & Vanaman, T. C. (1982) *Adv. Protein Chem. 35*, 213–321.

Klee, C. B., Crouch, T. H., & Richman, P. G. (1980) *Annu. Rev. Biochem. 49*, 489–515.

Laemmli, V. K. (1970) *Nature (London) 277*, 680.

LaPorte, D. C., & Storm, D. R. (1978) *J. Biol. Chem. 253*, 3374–3377.

LaPorte, D. C., Toscano, W. A., & Storm, D. R. (1979) *Biochemistry 18*, 2820–2825.

LaPorte, D. C., Keller, C. H., Olwin, B. B., & Storm, D. R. (1981) *Biochemistry 20*, 3965–3972.

Olwin, B. B., Keller, C. H., & Storm, D. R. (1982) *Biochemistry 21*, 5669–5675.

Peterson, G. L. (1977) *Anal. Biochem. 83*, 346.

Teshima, Y., & Kakiuchi, S. (1974) *Biochem. Biophys. Res. Commun. 56*, 489–495.

Wang, J. H., & Waisman, D. M. (1979) *Curr. Top. Cell. Regul. 15*, 47–107.

Watterson, D. M., & Vanaman, T. C. (1976) *Biochem. Biophys. Res. Commun. 73*, 40–46.

Westcott, K. R., LaPorte, D. C., & Storm, D. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 204–208.

Phosphonamidates as Transition-State Analogue Inhibitors of Thermolysin†

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ABSTRACT: Six phosphorus-containing peptide analogues of the form Cbz-$NHCH_2PO_2^-$-L-Leu-Y (Y = D-Ala, NH_2, Gly, L-Phe, L-Ala, L-Leu) have been prepared and evaluated as inhibitors of thermolysin. The K_i values for these compounds range from 1.7 μM to 9.1 nM and correlate well with the K_m/k_{cat} values for the corresponding peptide substrates [Morihara, K., & Tsuzuki, H. (1970) *Eur. J. Biochem. 15*, 374–380] but not with the K_m values alone. The correlation noted between inhibitor K_i and substrate K_m/k_{cat} is the most extensive one of this type, providing strong evidence that the phosphonamidates are transition-state analogues and not simply multisubstrate ground-state analogues. Cbz-$NH_2CH_2PO_2^-$-L-Leu-L-Leu (K_i = 9.1 nM) is the most potent inhibitor yet reported for thermolysin.

The concept of transition-state analogues (TS analogues)[1] is a successful one for the design of potent enzyme inhibitors (Wolfenden, 1976; Lienhard, 1973; Stark & Bartlett, 1983). The rationale for this approach is the recognition that addi-

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[1] Abbreviations: TS, transition state; M-S, multisubstrate; Cbz, benzyloxycarbonyl; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; FAGLA, 3-(2-furylacryloyl)glycyl-L-leucinamide.

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