Murry-Brelier, A., & Goldberg, M. E. (1988) *Biochemistry 27*, 7633-7640.
Murry-Brelier, A., & Goldberg, M. E. (1989) *Proteins: Struct., Funct., Genet. 6*, 395-404.
Onoué, K., Yagi, Y., Grossberg, A. L., & Pressman, D. (1965) *Immunochemistry 2*, 401-415.
Zetina, C. R., & Goldberg, M. E. (1980) *J. Mol. Biol. 137*, 401-414.
Zetina, C. R., & Goldberg, M. E. (1982) *J. Mol. Biol. 157*, 133-148.

Functional Sites of Glia-Derived Nexin (GDN): Importance of the Site Reacting with the Protease

Hanspeter Nick,*,‡ Jan Hofsteenge, Elliott Shaw, Giorgio Rovelli, and Denis Monard
Friedrich Miescher Institute, P.O. Box 2543, 4002 Basel, Switzerland
Received July 28, 1989; Revised Manuscript Received October 10, 1989

ABSTRACT: Glia-derived nexin (GDN) is a 43-kDa serine protease inhibitor with neurite promoting activity in mouse neuroblastoma cells (Guenther et al., 1985). In chick sympathetic neurons, GDN but not hirudin and synthetic peptide inhibitors promoted neurite outgrowth (Zurn et al., 1988). Thus, it was considered that the protease inhibitory activity cannot account for the total biological activity of GDN. We show here that synthetic peptide inhibitors with thrombin specificity mimic GDN at similar concentrations in neuroblastoma cells. Limited proteolysis of GDN with elastase causes a cleavage between sites P_1 and P_2, corresponding to residues Ala-344-Arg-345 of the molecule. The resulting fragments still copurify on heparin-Sepharose, but the protease inhibitor activity of GDN and the GDN neurite promoting activity are lost. The results confirm the necessity of an intact reactive site for the biological activity of GDN.

The function of glial cells is poorly understood although they are believed to play a key role in the differentiation of neurons. Cultured glial cells release a protein which promotes the extension of neurites in both mouse neuroblastoma cells (Monard et al., 1973) and primary cultures of chick sympathetic neurons (Zurn et al., 1988); furthermore, this glia-derived nexin (GDN)[1] affects the migration of granule cells (Lindner et al., 1986). In vivo, GDN is mainly detected in the olfactory system known for its constant degeneration and regeneration of neuronal cells (Reinhard et al., 1988). It is possible that this protein could act as an important mediator between glial cells and neurons.

The glia-derived neurite promoting factor is a 43-kDa protein consisting of a single polypeptide chain (Guenther et al., 1985). Kinetic studies have demonstrated that it is a potent inhibitor of thrombin, trypsin, and, to a lesser extent, urokinase (Stone et al., 1987). Complexes between the inhibitor and these proteases are resistant to SDS treatment and, therefore, can be visualized by SDS-PAGE. Heparin increases the rate at which GDN reacts with thrombin by over 40-fold to 8.9 $\times 10^{-7}$ M^{-1} s^{-1} and decreases the dissociation constant of the complex by over 80-fold to 0.3 pM. Sequence analysis of the rat and human cDNA clones coding for these inhibitors reveals that they belong to the serpin family (Gloor et al., 1986; Sommer et al., 1987). Results published recently show that the primary structure of protease nexin I, a serine protease inhibitor released by human fibroblasts, is identical with human GDN (McGrogan et al., 1988).

GDN contains at least two functional sites: a reactive center which interacts with the protease and a heparin binding site. Data presented here show that, through proteolytic modification close to the reactive center of the inhibitor, its contribution to the overall properties of the native molecule can be elucidated.

Hirudin, an inhibitor of thrombin, has been shown to be as potent as GDN in promoting neurite extension in neuroblastoma cells (Monard et al., 1983). To determine the relevance of protease inhibition for neurite extension, we have tested the effect of other naturally occurring protease inhibitors as well as that of synthetic Arg chloromethyl ketones on their ability to promote neurite extension in neuroblastoma cells. In addition, we show that the integrity of the protease inhibitory site of GDN has to be maintained for neurite promoting activity.

Materials and Methods

All reagents were analytical grade if not otherwise stated. DMEM and MEM powder media were from Gibco, and fetal calf serum was from North American Biological Incorp. Spectra/Por 2 molecular weight cutoff 12 000-14 000 dialysis membranes were from Spectrum Medical Industries Inc. The thrombin chromogenic substrate S-2888 was from Kabi, Sweden. α_2-Macroglobulin and secretory leukocyte proteinase inhibitor were a gift from Dr. Schnebli, Ciba-Geigy, Basel. Antithrombin III, aprotinin, leupeptin, ovomucoid, soybean trypsin inhibitor, and α_1-proteinase inhibitor were from Sigma. The chloromethyl ketones were synthesized as described (Kettner & Shaw, 1981; Walker et al., 1985).

Purification of GDN was carried out as described earlier (Guenther et al., 1985).

Elastase Digestion of GDN. GDN (2 mg in 1 mL) was incubated for up to 160 min at 37 °C in 50 mM Tris-HCl, pH 8 (Tris buffer), containing 400 mM NaCl with porcine elastase (Merck) at a protein to enzyme ratio of 50:1.

* To whom correspondence should be addressed.
‡ Present address: Ciba Geigy Ltd., K-125. 1.13, 4002 Basel, Switzerland.

[1] Abbreviations: GDN, glia-derived nexin; DMEM, Dulbecco's modified Eagle's medium; MEM, minimum essential medium Eagle, Tris, tris(hydroxymethyl)aminomethane; EGDN, elastase-cleaved glia-derived nexin; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin; SLPI, secretory leukocyte protease inhibitor.

0006-2960/90/0429-2417$02.50/0

Heparin–Sepharose chromatography following elastase digestion of GDN was performed essentially as described (Guenther et al., 1985). The incubation mixture was diluted with 3 mL of Tris buffer and added to 10 mL of heparin–Sepharose, stirred for 15 min at room temperature, and left overnight at 4 °C. The slurry was packed into a glass column and the gel washed with 30 mL of Tris buffer and then with 30 mL of 0.4 M NaCl in the same buffer. Elastase-cleaved GDN (EGDN) was eluted with 0.8 M NaCl in Tris buffer.

Reversed-Phase HPLC Chromatography of EGDN. Separation of 500 μg of EGDN was performed with a Vydac C4 wide-pore 0.46 × 25 cm column. Elution was with a linear gradient of acetonitrile in 0.1% TFA in water. Buffer A was 0.1% TFA in water; buffer B was aqueous 50% acetonitrile containing 0.1% TFA. The gradient was 0–100% buffer B in 90 min, starting 5 min after injection.

Neurite Outgrowth Bioassay. The neurite promoting activity of the various compounds was assayed as described earlier (Schürch-Rathgeb & Monard, 1978). For each compound, a dose response curve was established. The concentration giving half-maximal effect was used as a measure of its neurite promoting activity.

Monitoring GDN Inhibitory Activity with Thrombin and Chromozyme S-2288. Thrombin was isolated as described (Hofsteenge et al., 1986). The assays were performed in polystyrene cuvettes at 37 °C in 0.05 M Tris-HCl buffer, pH 7.8, which contained 0.1% poly(ethylene glycol) (PEG 6000), 0.1 mg/mL bovine serum albumin, and 0.1 M NaCl. Thrombin (1.1 pM) was preincubated at 37 °C with increasing amounts of GDN (or elastase-treated GDN) for 1 h. The substrate chromozyme S-2288 (H-D-Ile-L-Pro-L-Arg-*p*-nitroanilide dihydrochloride) was then added at a final concentration of 200 μM. The remaining thrombin activity was determined by the increase in absorbance at 405 nm.

SDS–PAGE (Laemmli, 1970) was performed in a slab gel (140 × 85 × 1.5 mm). The gel was overlaid with approximately 5 mm of stacking gel and run at 30 mA. Bio-Rad low molecular weight markers were used as standards. Proteins were visualized by silver staining (Eschenbruch & Buerk, 1982). Bio-Rad electrophoretic purity reagents were used except for the Tris.

Immunoblots. Proteins were transferred from slab gels to a nitrocellulose sheet (Bio-Rad, Trans-Blot transfer medium) using a buffer containing 3 g of Tris, 14.4 g of glycine, 100 mL MeOH, and 0.1 g of SDS per liter. The transfer was performed at room temperature for 75 min at 350 mA in a Bio-Rad TRANS BLOT cell.

After transfer, the nitrocellulose was blocked with 3% BSA for 1 h at room temperature. Proteins were visualized after an overnight incubation with polyclonal antibodies (3 μg/mL Tris buffer) which were raised in rabbits and purified on protein A–Sepharose (Gloor et al., 1986), followed by a 2-h incubation with a goat anti-rabbit horseradish peroxidase conjugate (BRL) diluted as suggested by the supplier. Visualization was performed with chloronaphthol as described (Towbin et al., 1979).

Protein Sequence Analysis. Automated Edman degradation was performed on an Applied Biosystems Model 470A gas–liquid-phase sequencer. PTH-amino acids recovered at each cycle of the Edman degradation were analyzed on-line with an Applied Biosystems Model 120A PTH-amino acid analyzer.

Results

Promotion of Neurite Extension by Protease Inhibitors. Hirudin is as potent as GDN in promoting neurite extension in neuroblastoma cells (Monard et al., 1983). This property and the fact that both are potent thrombin inhibitors suggest first that inhibiting proteolytic activity may be important for neurite extension in neuroblastoma cells and second that the enzyme to be inhibited may be thrombin or a protease with thrombin-like specificity. To test this possibility, Arg chloromethyl ketones, of which the reaction kinetics with thrombin are known, as well as other natural protease inhibitors were tested for their neurite promoting activity (Table I). α_2-Macroglobulin, antithrombin III (without heparin present), and soybean trypsin inhibitor are relatively poor inhibitors of thrombin and show marginal neurite promoting activity in neuroblastoma cells. By contrast, the Arg chloromethyl ketones that were the best inhibitors of thrombin were also the most potent agents in promoting neurite extension in neuroblastoma cells. It is interesting that secretory leukocyte protease inhibitor (SLPI) which is a potent inhibitor of human neutrophilic elastase and bovine pancreatic trypsin (Smith et al., 1985) but not of thrombin (unpublished results) is unable to promote neurite extension in neuroblastoma cells. This indicates that it is not the trypsin-like inhibitory activity of GDN but rather its antithrombin activity which is responsible for neurite promotion.

Table I: Neurite Promoting Activity of Protease Inhibitors in Neuroblastoma Cells

protease inhibitor	concn for half-maximal neurite outgrowth (M)
glia-derived nexin	5×10^{-10}
hirudin	5×10^{-10}
α_2-macroglobulin	5×10^{-8}
antithrombin III	5×10^{-7}
soybean trypsin inhibitor	5×10^{-7}
α_1-proteinase inhibitor	2×10^{-5}
ovomucoid	2×10^{-5}
aprotinin	6×10^{-5}
leupeptin	1×10^{-3}
SLPI	1×10^{-5}
EGDN	no outgrowth at 1×10^{-8}
D-Phe-Pro-Arg-CH_2Cl	5×10^{-10}
D-Tyr-Pro-Arg-CH_2Cl	1×10^{-10}
eBz-D-Lys-Pro-Arg-CH_2Cl	1×10^{-9}
Ile-Pro-Arg-CH_2Cl	5×10^{-7}
Val-Ile-Pro-Arg-CH_2Cl	5×10^{-6}
Val-Pro-Arg-CH_2Cl	5×10^{-6}
DNS-Glu-Gly-Arg-CH_2Cl	1×10^{-6}
D-Tyr-Gly-Arg-CH_2Cl	no outgrowth at 1×10^{-8}
D-Tyr-Phe-Arg-CH_2Cl	no outgrowth at 1×10^{8}

Elastase Digestion of GDN. In order to demonstrate that only GDN with an intact inhibitory site has the ability of promoting neurite extension, enzymatic modification of the native molecule was attempted. Its sensitivity to elastase digestion was investigated: the amino acid sequence derived from cDNA (Sommer et al., 1987) had revealed the presence of a potential elastase-susceptible bond close to the reactive site. Limited proteolysis by elastase, followed by SDS–PAGE, showed a striking resistance to degradation by this protease despite the fact that many potential cleavage sites are present. In fact, after a 3-h incubation, only one new protein band appears (Figure 1A, lane 2). This breakdown product of GDN has an apparent molecular weight of approximately 40K. A good separation of cleaved and uncleaved form was obtained under nonreducing conditions in SDS–PAGE (Figure 1B). The band pattern of the elastase-treated GDN was similar to the pattern one often observes with protease-complexed forms of GDN (Figure 1C). Complexes of GDN with thrombin (and also with trypsin) seem to yield a clipped form of GDN with a molecular weight comparable to that observed with the elastase-digested molecule.

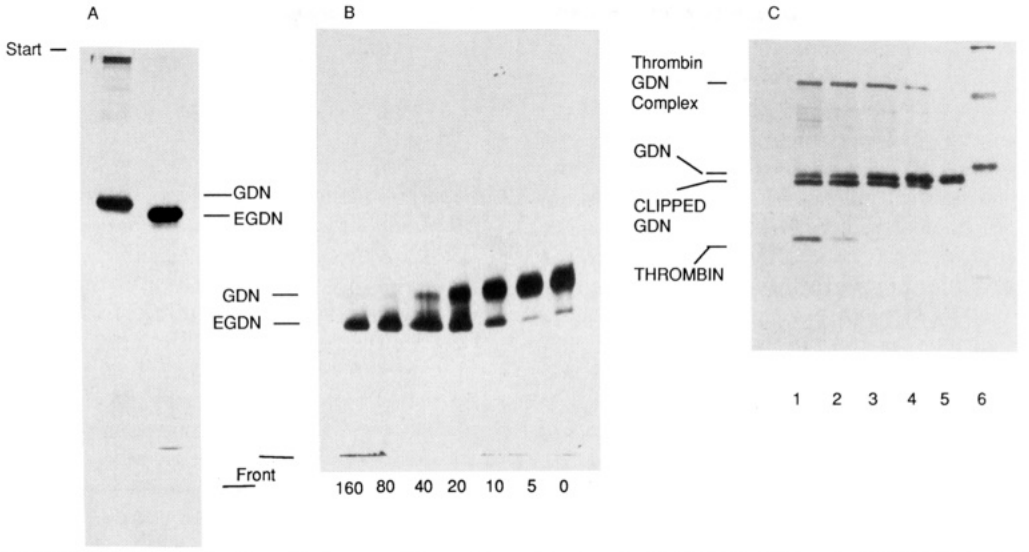

FIGURE 1: (A) SDS–PAGE, 12% acrylamide, reducing conditions, silver stain: lane 1, GDN (0.5 μg); lane 2, EGDN (0.5 μg). (B) SDS–PAGE, 10% acrylamide, nonreducing conditions, silver stain. Time course of GDN digestion with elastase: 0, 5, 10, 20, 40, 80, and 160 min of digestion (0.5 μg per lane). (C) SDS–PAGE, 10% acrylamide, reducing conditions, silver stain. Complex formation of GDN with decreasing amounts of thrombin. The ratios of thrombin to GDN were 5:1, 2:1, 1:1, and 0.5:1 and GDN only (lanes 1, 2, 3, 4, and 5, respectively). The bands in lane 6 represent (from top to bottom) phosphorylase a_2 (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), and soybean trypsin inhibitor (M_r 20 100). Incubation, before adding sample buffer containing SDS and mercaptoethanol, was at 37 °C for 20 min.

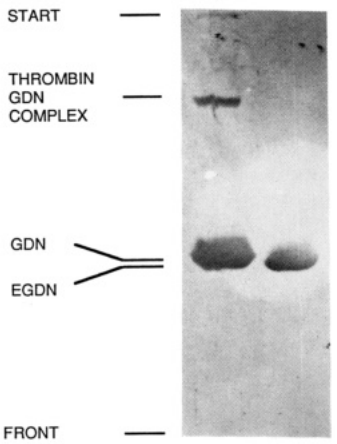

FIGURE 2: Immunoblot, GDN (2 μg, lane 1) and EGDN (2 μg, lane 2), was incubated with thrombin (0.5 μg) for 20 min at 37 °C followed by SDS–PAGE and blotting. GDN staining was as described under Materials and Methods. A similar experiment (not shown) was carried out with biotinylated thrombin instead of thrombin, showing that biotinylated thrombin did form complexes with GDN but did not with EGDN.

Properties of Elastase-Digested GDN. GDN treated with elastase (EGDN) retains its heparin binding property. After an overnight incubation of the reaction mixture with heparin–Sepharose in 50 mM Tris-HCl and 0.1 M NaCl, pH 8, only a trace amount of the digested material remained in the supernatant, as determined by SDS–PAGE. EGDN could be eluted from the heparin–Sepharose with 0.8 M NaCl. The eluted material was no longer capable of inhibiting the amidolytic activity of thrombin (results not shown), and complex formation with thrombin was not observed (Figure 2). Moreover, even at a concentration of 10^{-8} M, EGDN was not able to promote neurite extension in neuroblastoma cells (Table I). When subjected to RP-HPLC without prior reduction and carboxymethylation, the material emerged in two well-separated peaks eluting at 72 and 87 min (results not shown). These fractions were further analyzed by Edman degradation on a gas-phase sequencer. A single sequence was established from material corresponding to the earlier eluting peak (Figure 3). It could be unequivocally placed within the protein sequence derived from the cDNA. The elastase-susceptible bond was identified as that between the alanyl and arginyl residues which represent the P_2 and P_1 positions of the inhibitor, respectively (Gloor et al., 1986; Sommer et al., 1987). The bulk of the material, which elutes later on the RP-HPLC column, was resistant to Edman degradation, suggesting the presence of a blocked N-terminus. Since native GDN was also resistant to Edman degradation, the second peak is believed to represent the N-terminal region of the molecule up to the P_2 residue. This suggests that the Ala–Arg bond close to the active site is the most susceptible bond to elastase cleavage in GDN.

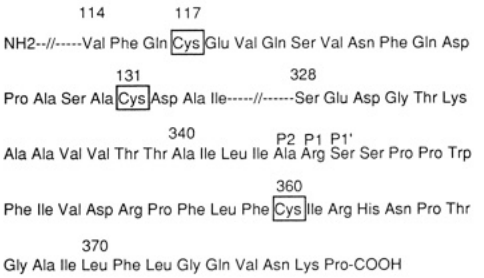

FIGURE 3: Partial sequence of rat GDN. The C-terminal peptide sequenced is from position 345 (P_1, Arg) to position 375 (Val). Cysteines are boxed. Cysteine-117 and cysteine-131 of the rat sequence are at corresponding positions in human GDN, whereas cysteine-360 is not conserved in position in the human sequence.

Discussion

GDN is a serine protease inhibitor belonging to the serpins. These are related protease inhibitors which have diverged from a common ancestral protein over 500 million years ago (Doolitle, 1983). The reactive center of GDN can be identified by aligning its sequence with that of other known serpins (Sommer et al., 1987); it possesses an Arg residue at position P_1 (Argserpin) and consequently inhibits trypsin-like enzymes. Since GDN is a potent promoter of neurite extension, it was of interest to determine whether the inhibitory activity and neurite promoting activity were connected or whether these were separate properties of the molecule. Two lines of evidence indicate that the inhibitory activity is necessary for promoting neurite extension in neuroblastoma cells. First, the elastase-

clipped form of GDN, EGDN, neither inhibits thrombin nor promotes neurite extension. Second, other protease inhibitors that react well with thrombin also promote neurite extension, at least in neuroblastoma cells.

EGDN eluted from heparin–Sepharose showed two peaks on RP-HPLC which can be explained as (a) either these fragments bind independently to heparin–Sepharose or, more likely (b) these fragments are noncovalently linked together even in the presence of a washing buffer which contains 0.4 M NaCl. The conditions required for the separation of the two fragments on HPLC modify properties of these polypeptides. Experiments aiming at the elucidation of this phenomenon were not followed up since this problem does not interfere with our conclusions.

Similarly, a noncovalent interaction between a C-terminal peptide and the rest of the protein was observed with α_1-proteinase inhibitor (α_1-PI) after cleavage of the molecule with chymotrypsinogen. Cleavage of α_1-PI occurred between residues P_1 and P_1', and the newly created C-terminal peptide remained noncovalently attached to the rest of the molecule (Loebermann et al., 1982).

Limited treatment of GDN with elastase results in the cleavage of the peptide bond between P_1 and P_2 as determined by Edman degradation. The early eluting RP-HPLC peak represents the C-terminal portion of the molecule. The bulk of the material eluted later and resisted degradation by the Edman procedure, in line with results obtained using intact GDN (data not shown). This is in contrast to results obtained with protease nexin I of which the 21 N-terminal amino acid residues were susceptible to Edman degradation (Scott et al., 1985). This may be due to different posttranslational modifications of the two proteins which are derived from different types of cells and reveal distinct molecular weights on SDS-PAGE. Though unlikely, the difference at the amino terminus could also be explained by variations in the isolation procedures adopted, perhaps leading to a blocked N-terminus in GDN.

As demonstrated, there is one prominent site in rat GDN susceptible to porcine pancreatic elastase cleavage. The band pattern on SDS–PAGE seen after the elastase digestion bears striking similarities to that obtained when GDN is incubated with thrombin or trypsin (Figure 1C). A slow cleavage occurring at the inhibitory site after complexation may explain the appearance of a slightly faster migrating polypeptide which is identical in size with EGDN. The reactive site of serine protease inhibitors is expected to be on a loop exposed on the surface of the molecule in a way that it would fit into the specificity pocket of the target enzyme. This may explain the relatively fast cleavage of GDN by elastase within this loop as compared to other sites. Also, a possible physiological regulatory function of the GDN inhibitory activity, for example, by leukocyte elastase, has to be envisaged.

Sequences of rat and human GDN have now been fully determined (Gloor et al., 1986; Sommer et al., 1987). Three cysteines were found in both sequences. If the sequences of rat and human GDN are aligned for optimal homology, it is evident that two cysteines (at positions 117 and 131) are at identical positions. In rat GDN, the conserved cysteines reside in the N-terminal segment (relative to the elastase cleavage site at P_2–P_1) whereas the third cysteinyl residue at position 360 occurs toward the C-terminus. By contrast, in human GDN, the third cysteinyl residue is located on the N-terminal side, at position 209. The C-terminal peptide created by elastase digestion of rat GDN could be separated by RP-HPLC from the rest of the molecule without prior reduction. This indicates that cysteine-360 (Figure 3) is not linked by a disulfide bond to one of the other two cysteines. Experiments in progress should establish whether the conserved cysteines (Cys-117 and -131) are connected by a disulfide bridge.

Since EGDN has lost both thrombin inhibitory and neurite promoting activity, the integrity of the reactive site is required to promote neurite extension in neuroblastoma cells. An intact heparin binding site alone is not sufficient to promote neurite extension. Since GDN acts as a very potent trypsin inhibitor, the effect of another trypsin inhibitor, i.e., the secretory leukocyte protease inhibitor, was tested for promotion of neurite extension. It was without effect, further supporting the hypothesis that inhibition of thrombin activity rather than inhibition of trypsin activity may be the triggering mechanism for neurite sprouting in neuroblastoma cells. Thrombin can abolish the GDN neurite promoting activity or cause a retraction of neurites in the mouse neuroblastoma cell clone NB_{2a} (Monard et al., 1983; Gurwitz & Cunningham, 1988). In the in vitro cellular system, GDN could simply act by inhibiting residual traces of thrombin due to previous incubation in the presence of serum, thus creating conditions suitable for better neurite adhesion and sprouting. It could as well suggest that excess of thrombin or thrombin-like proteases is nonpermissive for neurite outgrowth in vivo, especially where increased proteolytic activities are detected following axonal lesions (Bignami et al., 1982) or expected in the vicinity of angiopathic brain capillaries. The occurrence and the role of GDN in such in vivo systems are presently under investigation.

Hirudin and the synthetic peptide inhibitors of thrombin specifically mimic GDN in neuroblastoma cells but not in primary cultures of neuronal cells (Zurn et al., 1988). Likewise, and unlike GDN, they are also unable to influence the migration of granule cells monitored in cultured explants of cerebellum (Lindner et al., 1986). Thus, the protease inhibitory activity alone is not sufficient to explain the effect of GDN in these primary culture systems. Recent findings demonstrate that GDN as well as GDN complexed to thrombin binds to components of the extracellular matrix (Rovelli et al., submitted for publication). Taken together, these data could mean that, in neuroblastoma cells, the inhibition of proteolytic activity is sufficient to lead to the increased adhesion supported by other cell surface components. In normal neuronal cells, GDN, especially following its complexation with a protease, exposes epitopes which afford a level of adhesiveness permissive for the promotion of neurite outgrowth.

References

Bignami, A., Cella, G., & Chi, N. H. (1982) *Acta Neuropathol. 58*, 224–228.

Doolitle, R. F. (1983) *Science (Washington, D.C.) 222*, 417–419.

Eschenbruch, M., & Buerk, R. R. (1982) *Anal. Biochem. 125*, 96–99.

Gloor, S., Odink, K., Guenther, J., Nick, H., & Monard, D. (1986) *Cell 47*, 687–693.

Guenther, J., Nick, H., & Monard, D. (1985) *EMBO J. 4*, 1963–1966.

Gurwitz, D., & Cunningham, D. D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3440–3444.

Hofsteenge, J., Taguchi, H., & Stone, S. (1986) *Biochem. J. 237*, 243–251.

Kettner, C., & Shaw, E. (1981) *Methods Enzymol. 80*, 826–842.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lindner, J., Guenther, J., Nick, H., Zinser, G., Antonicek, H., Schachner, M., & Monard, D. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4568–4571.

Loebermann, H., Tokuoka, R., Deisenhofer, J., & Huber, R. (1984) *J. Mol. Biol. 177*, 531–556.
McGrogan, M., Kennedy, J., Ping Li, M., Hsu, C., Scott, R. W., Simonsen, C. C., & Baker, J. B. (1988) *Bio/Technology 6*, 172–177.
Monard, D., Solomon, F., Rentsch, M., & Gysin, R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1894–1897.
Monard, D., Niday, E., Limat, A., & Solomon, F. (1983) *Prog. Brain Res. 58*, 359–363.
Reinhard, E., Meier, R., Halfter, W., Rovelli, G., & Monard, D. (1988) *Neuron 1*, 387–394.
Schürch-Rathgeb, Y., & Monard, D. (1978) *Nature 273*, 308–309.
Scott, R. W., Bergmann, B. L., Bajpai, A., Hersh, R. T., Rodriguez, H., Jones, B. N., Barreda, C., Watts, S., & Baker, J. B. (1985) *J. Biol. Chem. 260*, 7029–7034.
Smith, C. E., & Johnson, D. A. (1985) *Biochem. J. 225*, 463–472.
Sommer, J., Gloor, S., Rovelli, G. F., Hofsteenge, J., Nick, H., Meier, R., & Monard, D. (1987) *Biochemistry 26*, 6407–6410.
Stone, S., Nick, H., Hofsteenge, J., & Monard, D. (1987) *Arch. Biochem. Biophys. 252*, 237–244.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Walker, C., Wilkstrom, C., & Shaw, E. (1985) *Biochem. J. 230*, 645–650.
Zurn, A. D., Nick, H., & Monard, D. (1988) *Dev. Neurosci. 10*, 17–24.

Allosteric Effects Acting over a Distance of 20–25 Å in the *Escherichia coli* Tryptophan Synthase Bienzyme Complex Increase Ligand Affinity and Cause Redistribution of Covalent Intermediates†

Karl F. Houben‡ and Michael F. Dunn*
Department of Biochemistry, University of California at Riverside, Riverside, California 92521
Received June 26, 1989; Revised Manuscript Received October 26, 1989

ABSTRACT: The reactions of L-histidine (L-His) and L-tryptophan (L-Trp) with the $\alpha_2\beta_2$ complex of *Escherichia coli* tryptophan synthase are introduced as probes both of β-subunit catalysis and of ligand-mediated α–β allosteric interactions. Binding of DL-α-glycerol 3-phosphate (GP), an analogue of 3-indole-D-glycerol 3′-phosphate (IGP), to the α-catalytic site increases the affinity of $\alpha_2\beta_2$ for L-His 4.5-fold and the affinity for L-Trp 17-fold and brings about a redistribution of β-bound intermediates that favors the quinonoids derived from each amino acid. Inorganic phosphate (P_i) (presumably via binding to the α-catalytic site) influences the distribution of L-His intermediates as does GP. Previous binding studies [Heyn, M. P., & Weischet, W. O. (1975) *Biochemistry 14*, 2962–2968] indicate that when the phosphoryl group subsite of the α-catalytic site is occupied by GP or P_i, a high-affinity indole subsite is induced at the α-catalytic site. Interaction of benzimidazole (BZ), an analogue of indole, with this site also shifts the distribution of β-bound L-His intermediates in favor of the L-His quinonoid. In the absence of P_i or GP, BZ interacts primarily at the β-catalytic site and competes with L-His for the β-subunit indole subsite. Since L-His and GP (or P_i) are substrate analogues and L-Trp is the physiological product, these allosteric effects likely take place with the natural substrates. Accordingly, the β-site becomes a higher affinity site for L-Ser, and L-Ser is in a more chemically reactive form when IGP (or D-glyceraldehyde 3-phosphate) is bound at the α-catalytic site. Hence, we postulate that, in vivo, ligand binding at the α-catalytic site confers changes to the β-catalytic site which increase the probability of L-Trp formation via an increased likelihood of L-Ser condensation with indole.

In biological systems, the control of protein function via allosteric regulation involves binding interactions at loci on the protein surface which bring about a change in conformation and a consequent change in properties at other loci. Most hypotheses to explain the behavior of allosteric protein systems have been restricted to models concerned with the modulation of ligand affinity via the binding of homotropic and heterotropic effectors (Monod et al., 1965; Koshland et al., 1966). In this paper, we introduce evidence indicating that long-range heterotropic binding can also alter the chemical reactivities and thermodynamic stabilities of covalent intermediates.

Native tryptophan synthase from *Escherichia coli* (EC 4.2.1.20) is a bienzyme complex with subunit composition $\alpha_2\beta_2$ that catalyzes the last two steps in the biosynthesis of L-tryptophan (L-Trp)[1] (Kirschner et al., 1975a; Miles, 1979).

†This work was supported by NSF Grants DMB-84-08513 and DMB-87-03697.
*Address correspondence to this author at the Department of Biochemistry, University of California at Riverside, Riverside, CA 92521-0129.
‡Present address: Department of Biological Chemistry, University of California, Los Angeles, CA 90024-4280.

[1] Abbreviations: PLP, pyridoxal 5′-phosphate; L-His, L-histidine; L-Ser, L-serine; L-Trp, L-tryptophan; $\alpha_2\beta_2$, the native form of *E. coli* tryptophan synthase (EC 4.2.1.20); E(Ain-E) and E(Ain-K), internal ϵ-aminolysyl aldimine enolimine and ketoenamine tautomers, respectively, of enzyme-bound PLP; E(S), enzyme–L-His or –L-Trp Michaelis complexes; E(GD), geminal diamine intermediate formed in the reaction of either L-Trp or L-His with the internal aldimine form of enzyme-bound PLP; E(Aex-K) and E(Aex-E), external aldimine ketoenamine and enolimine tautomers, respectively, formed with L-Trp or L-His; E(Q), quinonoid intermediate; GP, DL-α-glycerol 3-phosphate; IGP, 3-indole-D-glycerol 3′-phosphate; BZ, benzimidazole; P_i, inorganic phosphate; G3P, D-glyceraldehyde 3-phosphate; AU, absorbance unit(s).

0006-2960/90/0429-2421$02.50/0