Photoaffinity Inhibition of Dipeptide Transport in *Escherichia coli*†

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ABSTRACT: A dipeptide containing a nitrene precursor, glycyl-4-azido-2-nitro-L-phenylalanine, has been synthesized. This compound is a photoaffinity inhibitor of dipeptide transport in *E. coli*. In the dark, the dipeptide is a reversible inhibitor of glycylglycine uptake by live *E. coli* W cells. The ^{14}C-labeled compound is a substrate for the transport system, with a K_m of 7 μM and V_{max} of 5×10^3 molecules cell^{-1} s^{-1} (compare 9 μM and 1×10^4 molecules cell^{-1} s^{-1}, respectively, for the transport of glycylglycine under the same conditions). When intact *E. coli* cells are photolyzed at ~350 nm in the presence of the photolabile dipeptide, their ability to transport either glycylglycine or unphotolyzed glycyl-4-azido-2-nitro-L-phenylalanine is irreversibly inhibited, but their ability to transport arginine is unaffected. The presence of glycylglycine in the medium during photolysis protects the cells against the light-dependent inactivation of dipeptide transport.

The interactions of peptides with various membrane-associated receptors are involved in many and diverse physiological phenomena. Of these, the transport of small peptide metabolites into a cell is conceptually one of the simplest.

Uptake of small peptides through the plasma membrane has been observed in a wide variety of cells, from bacteria (Payne & Gilvarg, 1971; Payne, 1976) to the brush border cells of mammalian intestinal epithelia (Silk, 1974; Matthews & Adibi, 1976). In this study we report the use of a peptide photoaffinity reagent as a probe of dipeptide transport in intact *E. coli* W cells.

Two classes of peptide transport systems have been distinguished in *E. coli*, one for dipeptides and another for oligopeptides (Payne & Gilvarg, 1968a). The dipeptide system recognizes only L,L dipeptides (Levine & Simmonds, 1962; Kessel & Lubin, 1963) with a free (Gilvarg & Katchalski, 1965) or at least ionizable (Payne, 1974) amino terminus, and a free carboxy terminus (Payne & Gilvarg, 1968a). In contrast, the oligopeptide transport system does not require a free carboxy terminus, but the system has a reduced affinity for peptides in which the terminal carboxyl group has been modified or deleted (Payne & Gilvarg, 1968a). Growth studies, primarily with *Lactobacilli* (Shankman et al., 1960, 1962), have been interpreted (Payne & Gilvarg, 1971) to show that the first two N-terminal residues of an oligopeptide must be L-amino acids and that D-amino acids can be tolerated in the third (or, presumably, a subsequent) residue. Size restriction by the oligopeptide transport system depends not on the number of amino acid residues, but on the total hydrodynamic volume of the peptide (Payne & Gilvarg, 1968b). Dipeptides are taken up via both the dipeptide and the oligopeptide systems, but oligopeptides are recognized only by the oligopeptide transport system (Payne, 1968). The recent discovery of multiple oligopeptide transport systems in *Salmonella typhimurium* (Jackson et al., 1976) suggests that there may be more than one transport system in either or both of the classes of peptide transport systems in *E. coli*.

In terms of metabolic coupling, the transport systems of *E. coli* fall into three general classes (Boos, 1974; Simoni & Postma, 1975): the phosphoenolpyruvate-sugar-phosphotransferases phosphorylate specific mono- and disaccharides during transport (Roseman, 1972; Simoni, 1972), while the other two classes apparently translocate their substrates with no net chemical change. The dehydrogenase-coupled transport systems require for activity only membrane-bound components. Isolated cytoplasmic membrane vesicles, when provided with a dehydrogenase substrate, establish an electrochemical proton gradient that drives these transport systems (Ramos & Kaback, 1977a,b). Shock-sensitive transport systems, the class into which the dipeptide transport system falls (Cowell, 1974), require not only membrane-bound components but also periplasmic binding proteins that are involved in the recognition of substrates. These systems appear to require ATP (Heppel, 1971; Lin, 1971), but their mode of energy utilization is complex and as yet incompletely elucidated (Ferenci et al., 1977).

Experimental Section

Materials

4-Azido-2-nitro-L-phenylalanine. Phe($4N_3$,$2NO_2$)·HCl[1] was synthesized by modifications of previously published procedures (Fahrenholz & Schimmack, 1975; Escher & Schwyzer, 1975). *p*-Amino-L-phenylalanine (5.0 g, 28 mmol) was dissolved in concentrated H_2SO_4 (15 mL) and the solution was cooled to −15 °C. Fuming nitric acid (1.8 mL, 43 mmol), previously clarified by treatment with urea and sparging with N_2 (Freeman & Shepard, 1963), was added to the stirred solution at −15 °C over 35 min. The reaction mixture was then allowed to warm to 0 °C over 1 h and then to 10 °C over 2 h, after which it was poured onto ice (500 g). Barium hydroxide (47 g) was added with vigorous stirring at room temperature, and the resulting suspension was stirred for several hours. The suspension was then warmed to 70–80 °C and filtered, and the precipitate washed twice with further portions (500 mL) of hot water. The filtrates were pooled and decolorized with activated charcoal, and the solution was reduced in volume on a rotary evaporator and left to crystallize at 0 °C. Recrystallization from water–isopropyl alcohol gave 2.3 g (37%) of 4-amino-

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[1] Abbreviations used: Phe($4N_3$,$2NO_2$), 4-azido-2-nitro-L-phenylalanine; Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl; TLC, thin-layer chromatography.

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2-nitro-L-phenylalanine. TLC gave R_f in system A (silica plates eluted with 1-butanol:acetic acid:water, 90:10:25 by volume) 0.16, and R_f in system B (silica plates eluted with chloroform:methanol:concentrated aqueous ammonia, 50:50:10 by volume) 0.39.

All the following synthetic steps were carried out in the dark or under dim incandescent lighting. 4-Amino-2-nitro-L-phenylalanine (1.1 g, 3.8 mmol) was dissolved in 6 N HCl (3.0 mL) and the solution was cooled to 0 °C. A solution of sodium nitrite (0.40 g, 5.8 mmol) in water (3.0 mL) was added at 0 °C over 15 min with constant stirring. After 10 min, a solution of sodium azide (0.39 g, 6 mmol) in water (3.5 mL) was added over 10 min at 0 °C. The reaction mixture was stirred for 20 min in the cold and then allowed to warm to room temperature. The reaction mixture was then frozen, and the fine precipitate that remained on thawing was isolated by filtration and washed with small amounts of cold water and ether. Recrystallization from isopropyl alcohol gave 0.55 g (39%) of 4-azido-2-nitro-L-phenylalanine hydrochloride as off-white crystals. TLC: R_f in system A, 0.30; R_f in system B, 0.47. In aqueous solution it is a very pale yellow, with λ_{max} 249 nm (ϵ 2.0×10^4) and 333 nm (ϵ 1.7×10^3). The infrared spectrum of this compound includes a strong azide band at 2120 cm^{-1}. Anal. Calcd for $C_9H_{10}N_5ClO_4$: C, 37.57; H, 3.51; N, 24.35; Cl, 12.32. Found: C, 37.24; H, 4.11; N, 23.95; Cl, 11.95.

Glycyl-4-azido-2-nitro-L-phenylalanine. To a solution of Phe($4N_3$,$2NO_2$)·HCl (0.24 g, 0.84 mmol) and triethylamine (0.2 mL, 1.4 mmol) in dimethoxyethane (5.0 mL, previously treated with basic alumina) was added Boc-glycine *N*-hydroxysuccinimide ester (0.23 g, 0.84 mmol) (Anderson et al., 1964). The reaction mixture was stirred overnight in the dark at room temperature. The solution was then acidified with aqueous citric acid and the product was extracted into ethyl acetate. The ethyl acetate solution was extracted into dilute aqueous triethylamine. The Boc dipeptide was then taken into ethyl acetate after reacidification. TLC: R_f in system A, 0.56; system B, 0.67 (Boc-Gly has R_f in system A, 0.56; system B, 0.46). The ethyl acetate solution was dried over anhydrous Na_2SO_4, and the dipeptide was deblocked by treatment with HCl gas over 1 h at room temperature. The product Gly-Phe($4N_3$,$2NO_2$)·HCl crystallized from this solution on cooling as light yellow needles (0.13 g, 45% from Phe($4N_3$,$2NO_2$)·HCl). TLC: R_f in system A, 0.20; system B, 0.45. The peptide is ninhydrin positive, giving a yellow spot characteristic of N-terminal glycine (Offord, 1969). The infrared spectrum includes a strong band at 2120 cm^{-1} ($-N_3$). Anal. Calcd for $C_{11}H_{13}N_6ClO_5$: C, 38.32; H, 3.81; N, 24.38; Cl, 10.28. Found: C, 38.21; H, 4.23; N, 24.06; Cl, 10.38.

[1,2-^{14}C]Glycyl-4-azido-2-nitro-L-phenylalanine. [U-^{14}C]Glycine (85 Ci/mol, from ICN Radiochemicals) (1.0 mCi, 12 μmol), as a dilute solution in 0.01 N HCl, was freeze concentrated (to about 0.5 mL) and titrated to pH 8.5–9.0 with triethylamine. 2-(*tert*-Butyloxycarbonyloxyimino)-2-phenylacetonitrile (Itoh et al., 1975) (60 μmol) was added in dioxane (0.5 mL), and the solution left at room temperature for 12 h. The mixture was washed with ether and ethyl acetate and then acidified with aqueous citric acid (5% w/v). The product, Boc-[1,2-^{14}C]glycine, was extracted into ethyl acetate. TLC autoradiography indicated that the product was of high purity, with no detectable contaminating glycine. The solvent was removed in a stream of N_2. Yield: 0.78 mCi (78%).

Boc-[1,2-^{14}C]glycine *N*-hydroxysuccinimide ester was synthesized by a modification of the procedure of Anderson et al. (1964). Boc-[1,2-^{14}C]glycine (0.78 mCi, 9.5 μmol) was dissolved in a solution of *N*-hydroxysuccinimide (1.7 mg, 15 μmol) in dimethoxyethane (0.1 mL, previously treated with basic alumina). To this solution was added dicyclohexylcarbodiimide (3.1 mg, 15 μmol) in dimethoxyethane (0.1 mL). After stirring the reaction mixture overnight, acetic acid (10 μmol) was added. The reaction mixture was then filtered into a suspension of Phe($4N_3$,$2NO_2$)·HCl (6 mg, 21 μmol) and triethylamine (5 μL, 36 μmol) in dimethoxyethane (0.1 mL). The precipitate (of dicyclohexylurea) was washed with dimethoxyethane (2 × 0.1 mL) and the washings were added to the new reaction mixture which was then stirred overnight. The solvent was removed in a stream of N_2. Aqueous citric acid (2 mL, of 5% w/v) was added to the resulting solid, and the solution was extracted into ethyl acetate (2 × 3 mL). TLC of this solution indicated that it contained Boc-[1,2-^{14}C]Gly and Boc-[1,2-^{14}C]Gly-Phe($4N_3$,$2NO_2$). The protected dipeptide was isolated by preparative TLC in system B. The yield was 0.18 mCi (23%). Most of the remaining radioactivity was recovered as Boc-Gly. The Boc dipeptide was deblocked as described above, and the free peptide was purified by preparative TLC in system B. The yield was 0.15 mCi (83%).

Methods

Growth and Harvest of Cells. E. coli W (ATCC no. 9637) were grown to late log phase in minimal medium A (Miller, 1972). Cells were harvested by centrifugation at $6 \times 10^3 g$, at 3 °C for 5–10 min. The cells were washed once in cold medium A containing chloramphenicol (0.3 g/L) and resuspended at 4–5 times the final growth concentration (i.e., 1–4 × 10^9 cells/mL) in medium A containing chloramphenicol. A sample of this final suspension was taken for a dilution plating assay. The cell suspension was kept on ice until use.

Transport Assays. Uptake of radioactive substrates was measured by a procedure based on that of Piperno & Oxender (1968). The stock cell suspension prepared as described above was incubated at 37 °C for 8–10 min before a set of transport assays was begun. For each assay, a portion (0.20 mL) of the stock suspension was transferred to a tube containing medium A with chloramphenicol plus the appropriate assay substrates (1.0 mL) at 37 °C. After 20 s, a portion (1.0 mL) of this suspension was pipetted onto a nitrocellulose filter (Millipore, 24-mm diameter, 0.45-μm pore size). The cells were then rapidly washed on the filter using medium A with chloramphenicol (5 mL) at room temperature, before scintillation counting. Assays were carried out in sets of 12, with individual assays 1 min apart. Peptide uptake was measured by counting the filters in a liquid scintillation counter. Adsorbed radioactivity was determined by assaying toluene-killed cells analogously (Cowell, 1974), and the uptake data were corrected for these backgrounds.

Photolyses. Photolyses of cell suspensions were carried out at 37 °C in the temperature-controlled apparatus previously described (Staros & Richards, 1974). A pair of General Electric 15T8-BLB fluorescent lamps was mounted laterally along the axis of the outer jacket of the chamber approximately 2 cm from the sample, and water at 37 °C was continuously pumped through the outer jacket.

A portion (2.5 or 3.5 mL) of stock cell suspension in medium A with chloramphenicol, prepared as described above, was transferred to the 13 × 100 mm inner tube at 0 °C. After addition of the test peptide(s) to the appropriate concentration(s), the tube was immediately sealed and mounted in the photolysis apparatus, the inner chamber was filled with water at 37 °C, and the motor to rotate the inner tube was switched on. One minute was allowed for temperature equilibration and then the sample was photolyzed for 1 min. The suspension was then diluted to 25 mL or 40 mL with fresh medium A with chloramphenicol at 0 °C; the cells were isolated by centrifugation

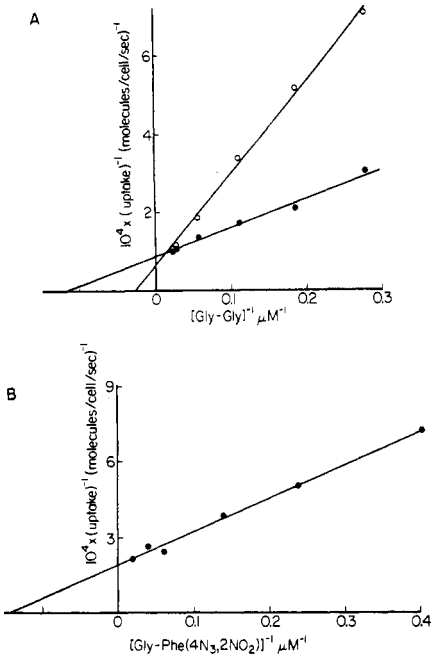

FIGURE 1: (A) Uptake of [^{14}C]Gly-Gly by *E. coli*. Uptake was followed in the absence (●) or in the presence (○) of Gly-Phe(4N$_3$,2NO$_2$) (31 μM). Each data point is the average of two assays from a single stock cell suspension. For further details, see the text. (B) Uptake of [^{14}C]Gly-Phe(4N$_3$,2NO$_2$) by *E. coli*. For details, see the text.

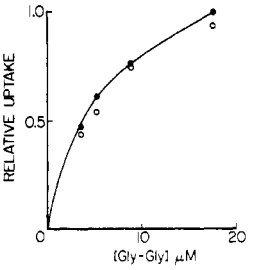

FIGURE 2: Effect of photolysis on [^{14}C]Gly-Gly uptake. Portions of cell suspension were either photolyzed (○) or incubated analogously in the dark (●). The cells were then washed once (2.5 mL of cell suspension was diluted to 25 mL in medium A, and the centrifuged cells were resuspended to 2.5 mL in medium A) and subjected to transport assays using [^{14}C]-Gly-Gly.

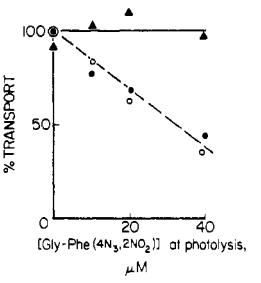

FIGURE 3: Photoinhibition of dipeptide uptake by Gly-Phe(4N$_3$,2NO$_2$). Portions of cell suspension were photolyzed in the presence of different concentrations of Gly-Phe(4N$_3$,2NO$_2$). The cells were then washed once (3.5 mL of cell suspension was diluted to 40 mL in medium A, and the centrifuged cells were resuspended to 3.5 mL in medium A) and subjected to transport assays using [^{14}C]Gly-Gly (●), unphotolyzed [^{14}C]Gly-Phe(4N$_3$,2NO$_2$) (○), and [^{14}C]Arg (▲). Each data point is the mean of two determinations. One hundred percent transport is defined as the uptake of peptide by cells photolyzed in the absence of Gly-Phe(4N$_3$,2NO$_2$), or for arginine, the average uptake under all conditions tested. Substrate concentrations for transport assays were 5 μM.

and then resuspended in medium A with chloramphenicol for the transport assay.

Results

When the transport of [1-^{14}C]Gly-Gly from the extracellular medium into *E. coli* is measured in the absence and in the presence of Gly-Phe(4N$_3$,2NO$_2$), the results are as shown in Figure 1A. We do not infer from the linear double-reciprocal plots that Gly-Gly enters via a single transport system since it is known that dipeptides compete for the oligopeptide transport system (Payne, 1968), and under somewhat different conditions, Gly-Gly uptake (i.e., transport) has been shown to exhibit biphasic character in double reciprocal plots (Cowell, 1974).

The inhibition of Gly-Gly transport by Gly-Phe(4N$_3$,2NO$_2$) is not cleanly competitive, and it appears that Gly-Gly may be taken up by a number of transport systems, and that the affinities of these systems for Gly-Gly and for Gly-Phe(4N$_3$,-2NO$_2$) differ. However, the data do indicate that Gly-Phe(4N$_3$,2NO$_2$) is a potent inhibitor of Gly-Gly uptake.

Gly-Phe(4N$_3$,2NO$_2$) is not only a reversible inhibitor of Gly-Gly uptake, it is also itself a substrate for transport. The results for the transport of [1,2-^{14}C]Gly-Phe(4N$_3$,2NO$_2$) are shown in Figure 1B. The K_m for Gly-Phe(4N$_3$,2NO$_2$) uptake is 7 μM (compared with 9 μM for Gly-Gly), and the V_{max} is 5×10^3 molecules cell^{-1} s^{-1} (compared with 1×10^4 molecules cell^{-1} s^{-1} for Gly-Gly).

It has been shown that irradiation of *E. coli* in the near ultraviolet, the useful region for photolysis of peptides containing Phe(4N$_3$,2NO$_2$), can inactivate certain transport systems (Koch et al., 1976). However, irradiation of cells for 1 min under the conditions described in Methods has a negligible effect on Gly-Gly uptake (Figure 2). When suspensions of cells are irradiated for 1 min in the presence of increasing concentrations of Gly-Phe(4N$_3$,2NO$_2$), there is an irreversible inactivation of the uptake of Gly-Gly or of unphotolyzed Gly-Phe(4N$_3$,2NO$_2$), while arginine uptake is unaffected (Figure 3). Arginine transport was chosen as the control because arginine, like dipeptides, is transported exclusively via a shock-sensitive transport system (Lombardi & Kaback, 1972) and is therefore likely to be metabolically coupled in the same way as is dipeptide transport. The observed photoinactivation of dipeptide transport is therefore likely to be due to an effect on the transport system per se rather than to some light-dependent metabolic uncoupling.

We have shown that the cells are fully functional with respect to dipeptide transport after photolysis alone, and fully functional with respect to arginine transport after photolysis in the presence of Gly-Phe(4N$_3$,2NO$_2$). Dilution plating assays of cells photolyzed in the presence and absence of Gly-Phe(4N$_3$,2NO$_2$) indicate that most of the cells were also viable after these treatments. The number of colony-forming cells after (a) no photolysis, (b) photolysis alone, and (c) photolysis in the presence of 20 μM Gly-Phe(4N$_3$,2NO$_2$) was $3.2 \pm 0.3 \times 10^9$, $3.2 \pm 0.7 \times 10^9$, and $2.6 \pm 0.3 \times 10^9$ cells/mL, respectively.

When Gly-Gly and Gly-Phe(4N$_3$,2NO$_2$) were added together to cell suspensions, and the suspensions photolyzed, it was evident that Gly-Gly protects against the photoinactivation by Gly-Phe(4N$_3$,2NO$_2$). Portions of cell suspension were photolyzed in the presence of Gly-Phe(4N$_3$,2NO$_2$) (20 μM) and different concentrations of Gly-Gly. After washing, the cells were subjected to transport assays using [^{14}C]Gly-Gly and [^{14}C]Arg (see legend to Figure 3). Cells photolyzed as above in the presence of 44 μM Gly-Gly, retained 18% of the ability to transport [^{14}C]Gly-Gly that was lost when cells were

photolyzed with Gly-Phe($4N_3$,$2NO_2$) alone. With 88 μM Gly-Gly, 39% was protected. Cells in all of these preparations took up [^{14}C]Arg at 100 ± 4% of cells in a control that was photolyzed in the absence of dipeptides, but otherwise handled as above.

Discussion

The design of an affinity reagent for a receptor that has a binding function but no catalytic function poses special problems. In the affinity labeling of an enzyme with a reactive substrate analogue, one usually exploits the high local concentration of the reagent in the vicinity of reactive residues at the active site to give the specificity required for active site labeling (Wold, 1977). In the case of suicide substrates, the site specificity is enhanced by the requirement that the enzyme activates the reagent by catalyzing an early step in the normal reaction (Rando, 1977). A noncatalytic receptor cannot, however, be expected to exhibit more than binding specificity towards added ligands, and the recognition site may well contain no chemically reactive functionalities. It is therefore necessary to invest in the affinity reagent sufficient reactivity to obtain a covalent adduct with relatively unreactive residues in the binding site of the receptor. Because of the high reactivity of some photochemically derived species, photoaffinity reagents (Singh et al., 1962) are good candidates for probing noncatalytic receptors. Photoaffinity reagents have the additional advantage that prior to photolysis they should be simple reversible inhibitors of the receptor sites at which they are aimed.

Aryl azides have proved very useful as the photoreactive moiety in photoaffinity probes (Bayley & Knowles, 1977). In the unphotolyzed state, aryl azides are unreactive molecules, but when photolyzed to aryl nitrenes, the nitrene may (among other things) insert into carbon–hydrogen bonds to form stable secondary aryl amines (Smith, 1970). For instance, it has been shown that an aryl nitrene, reacting in this manner, can covalently label alanyl and phenylalanyl residues in a receptor (Cannon et al., 1974). Recently it has been demonstrated that photolysis of aryl azides can also lead to 1-aza-1,2,4,6-cycloheptatetraenes, which can be attacked by nucleophiles, leading to azepines (Chapman & LeRoux, 1978).

Photoaffinity reagents have been used to probe many diverse biological systems (Bayley & Knowles, 1977); however, very few successful photoaffinity probes of peptide receptors have been reported. Escher & Schwyzer (1974, 1975) have reported that blocked peptides of the form Z-Ala-Ala-X [where X is a photolabile amino acid e.g., Phe($4N_3$,$2NO_2$)], can on photolysis irreversibly inhibit and covalently attach to chymotrypsin. Galardy & Jamieson (1975, 1977) and Das et al. (1977) have modified the amino termini of pentagastrin and of epidermal growth factor, respectively, with ligands containing aryl azides, and have used these derivatized peptides to photolabel pancreozymin and epidermal growth factor receptors. In the present work we have synthesized a peptide photoaffinity reagent in which the aromatic ring of Gly-Phe has been substituted so as to create a photolabile derivative. Peptide reagents of this type should prove useful as probes for a variety of peptide receptors where modification of the peptide termini or of charged side chains leads to loss of recognition by the receptor.

In order for a reagent to be a photoaffinity inhibitor of a receptor, the reagent should reversibly inhibit receptor function in the unphotolyzed state, and it should result in irreversible inhibition of the receptor function on photolysis. Further, the natural ligand for the receptor should protect against photoinactivation by the photolabile ligand.

Our results demonstrate that Gly-Phe($4N_3$,$2NO_2$) is a photoaffinity inhibitor of dipeptide transport in intact *E. coli*. The unphotolyzed peptide is a substrate for transport, with kinetic parameters for its uptake very close to those for the uptake of Gly-Gly. When a suspension of *E. coli* is photolyzed at ~350 nm while the cells are taking up Gly-Phe($4N_3$,$2NO_2$), the ability of the cells to transport dipeptides, i.e., Gly-Gly or unphotolyzed Gly-Phe($4N_3$,$2NO_2$), is irreversibly and specifically lost. This irreversible inactivation appears to be due to the reaction of the photolabile dipeptide specifically with the transport system(s) for dipeptides, since the uptake of arginine is unaffected by this treatment. Further, Gly-Gly protects the receptor against the photoinactivation by Gly-Phe($4N_3$,$2NO_2$).

We are currently extending these studies by utilizing [^{14}C]Gly-Phe($4N_3$,$2NO_2$), to identify individual components of the dipeptide transport system(s).

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Nuclear Magnetic Resonance Measurement of Hydrogen Exchange Kinetics of Single Protons in Basic Pancreatic Trypsin Inhibitor†

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ABSTRACT: Hydrogen-deuterium exchange rates of single protons assigned to peptide amide NH have been measured in basic pancreatic trypsin inhibitor (BPTI) by proton nuclear magnetic resonance spectroscopy. The pD dependence of exchange rates for 8 slowly exchanging protons was measured over the range of pD 2.1–7.2. For the slowly exchanging BPTI protons, pD_{min}, the pD at minimum rate, $\simeq$ 4, while in random coil polypeptides, pD_{min} = 2.5–3. The exchange rates at pD_{min} are four orders of magnitude less than those calculated for the random conformation of BPTI. The observed exchange with respect to D^+ and OD^- ions is not first order, as expected from model compounds; there is a change of a factor of 2.5–4 in exchange rate per pD unit. Taken together, the features of the pH dependence suggest that the exchange event is not occurring in bulk solvent. This supports a mechanism for the rate-limiting protein conformational process(es) involving multiple small amplitude protein fluctuations that accommodate the penetration of the solvent species into the interior of the folded protein. The temperature dependence was measured at pD 4.6, 52–70 °C, and at pD 7.25, 58–68 °C, with the observable temperature range at a single pD limited by the high activation energies of 49–89 kcal/mol ± 10%. This suggests that in BPTI these very slowly exchanging protons are in regions of the structure in which small displacements of the surrounding atoms have high energy barriers, and that each proton has a different average set of protein motions determining its exchange. One of the downfield exchangeable resonances may not be a peptide NH as it does not exhibit the characteristic doublet splitting from $NH-C_\alpha H$ coupling. For 7 of the 10 resonances observed over this pD range, the chemical shift changes <0.05 ppm. Three resonances shift upfield 0.05–0.1 ppm as the pD is raised from pD 2 to 7, indicating the proximity of a titrating group, probably carboxyl. A marked increase in resolution is observed below pD 3, suggesting a decrease in rotational correlation time at the lower pD, perhaps from dissociation.

The exchange rates of protein peptide amide protons with solvent are a measure of the accessibility of solvent species to the polypeptide backbone. The hydrogen exchange kinetics of a folded protein are many orders of magnitude slower than those of the random conformation of the same protein, in keeping with the solvent shielding expected in a solution structure that approximates the crystal structure. However, the existence of finite exchange rates for the great majority of exchangeable protons necessitates the modification of models derived from the crystal structure of a rigid, tightly packed, solvent impenetrable protein, to include dynamic processes for the solution structure of folded proteins. In many proteins, 80–90% of the exchangable protons exchange within 24 h under conditions in which the folded conformation of the protein is greatly favored (Woodward & Rosenberg, 1971; Wickett et al., 1974; Woodward et al., 1975; Ellis et al., 1975).

The interpretation of hydrogen exchange kinetics in terms of the rate-limiting protein conformational process(es) involves

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