J. Membrane Biol. 121, 201–214 (1991)
002226319100067G

The Journal of
Membrane Biology

Dissecting the Molecular Mechanism of Ion-Solute Cotransport: Substrate Specificity Mutations in the *putP* Gene Affect the Kinetics of Proline Transport

Richard S. Myers*, David Townsend**, and Stanley Maloy
Department of Microbiology, University of Illinois, Urbana, Illinois 61801

Summary. Rare mutations that alter the substrate specificity of proline permease cluster in discrete regions of the *putP* gene, suggesting that they may replace amino acids at the active site of the enzyme. If *putP* substrate specificity mutations directly alter the active site of proline permease, the mutants should show specific defects in the kinetics of proline transport. In order to test this prediction, we examined the kinetics of three *putP* substrate specificity mutants. One class of mutation increases the K_m over 120-fold but only decreases the V_{max} fourfold. Such K_m mutants may be specifically defective in substrate recognition, thus identifying an amino acid critical for substrate binding. Another class of mutation decreases the V_{max} 80-fold without changing the K_m. V_{max} mutants appear to alter the rate of substrate translocation without affecting the substrate binding site. The last class of mutation alters both the K_m and V_{max} of proline transport. These results indicate that substrate specificity mutations alter amino acids critical for Na^+/proline symport.

Key Words *putP* · substrate specificity · proline transport

Introduction

Solute transport is an essential and often rate-limiting step in a metabolic pathway. In *Salmonella typhimurium* and *Escherichia coli*, approximately 40% of all transport is catalyzed by ion/solute cotransport systems (permeases) (Wilson, 1978). However, very few permeases have been studied in detail, and the precise mechanism of substrate binding and translocation is not yet known for any transport system. The paradigm for ion-solute symport systems is the lactose permease of *E. coli*. Lactose permease has been the object of many elegant biochemical studies aimed at defining its structure and function. For example, chemical modification of lactose permease identified residues thought to be important for substrate binding and translocation (Fox & Kennedy, 1965). However, site-directed mutagenesis demonstrated that the modified residues were not required for galactoside transport, suggesting that chemical modification of the protein had an indirect effect on lactose permease activity (Trumble et al., 1984; Vitmen et al., 1985). For this reason, genetics may provide a more powerful approach for identifying active site residues than chemical modification. In addition, integral membrane proteins are difficult to work with, making dissection of permease structure and function by genetic approaches a promising alternative to biochemical approaches.

A variety of mutations that alter the specificity of permeases have been described recently (Mieschendahl et al., 1981; Niiya et al., 1982; Brooker & Wilson, 1985; Shiota et al., 1985; Dila & Maloy, 1986; Kaback, 1987; Botfield & Wilson, 1988; Myers & Maloy, 1988). In general, two approaches have been taken to isolate active site mutants: (*i*) random mutagenesis and (*ii*) site-directed mutagenesis. Using random mutagenesis, a large number of mutations that alter the substrate specificity of a permease can be isolated without prior identification of amino acids at the active site. By localized mutagenesis with selection for a marker located close to the locus under study, rare mutants can be obtained at a high frequency without directly selecting the mutant phenotype (Hong & Ames, 1971; Davis, Botstein & Roth, 1980). If a number of mutagens of different specificity are used, potentially all the amino acids that comprise the active site of the enzyme may be identified. In contrast, by site-directed mutagenesis the investigator predicts which amino acids will be important for catalysis, alters those codons in vitro, and measures the effect on enzyme activity. As an initial approach, site-directed mutagenesis is a labor-intensive method for identifying amino acids at the active site of an enzyme. However, once the active site has been identified by random mutagenesis, the number of residues to be tested by site-directed mutagenesis can be re-

* *Present address:* Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403.
** *Present address:* Department of Biological Sciences, Illinois State University, Normal, Illinois 61761.

Table 1. Bacterial strains used

Strain	Genotype[a]	Source
LT2	Wild type	J. Roth
MS689	*putP1024*::Mu*dJ mel B363*::Tn*10*	This study
MS691	*putP1155 proP*::Tn*10dCm zcc*−*7*::Tn*10*	This study
MS692	*putP1154 proP*::Tn*10dCm zcc*−*7*::Tn*10*	This study
MS695	*putP1160 proP*::Tn*10dCm zcc*−*7*::Tn*10*	This study
MS700	*proP*::Tn*10dCm zcc-7*::Tn*10*	This study
MS1209	*putP1024*::Mu*dJ*	Hahn et al. (1988)

[a] Genetic nomenclature is as described by Sanderson and Roth (1988). Nomenclature for Mu*d* insertions is as proposed by K. Hughes and J. Roth (*personal communication*): Mu*dJ* (Mu*dII734*) is a transposon that forms *lac* operon fusions.

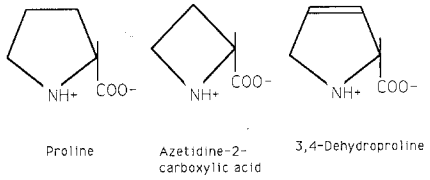

Fig. 1. Structures of proline permease substrates

duced to a managable number (Brooker & Wilson, 1985).

Proline permease, the product of the *putP* gene in *S. typhimurium,* is particularly amenable to genetic studies due to the availability of several proline analogs that are toxic to the cell (Maloy, 1987). Proline permease transports proline and proline analogs into cells by Na^+ symport. The wild type strain requires the *putP* permease for growth on proline as a sole source of nitrogen and sensitivity to three toxic proline analogs: 3,4-dehydroproline, azetidine-2-carboxylic acid, and thioproline (Fig. 1). By localized mutagenesis and screening for mutants that transport some, but not all of these substrates, a large number of mutants that alter the substrate specificity of proline permease were isolated (Dila & Maloy, 1986). Since *putP* substrate specificity mutations result in pronounced changes in transport of some substrates, without apparently altering transport of other substrates, these mutations may directly alter the active site of proline permease (Myers, Dila & Maloy, 1987).

If substrate specificity mutations are active site mutations, three predictions can be made. (*i*) Active site mutations should be recovered less frequently than simple structural mutations (such as null mutations). As predicted, *putP* mutations that alter the substrate or cation specificity of proline permease are recovered less than 1/10 as often as simple null mutations (Dila & Maloy, 1986; Myers & Maloy, 1988). (*ii*) Active site mutations should map to a limited portion of the gene, whereas null mutations should be found throughout the structural gene. As predicted, mutations that alter the substrate or cation specificity of proline permease cluster in small regions of the *putP* gene (Dila & Maloy, 1986; Myers & Maloy, 1988). (*iii*) Active site mutations should specifically alter the kinetics of catalysis.

In this study we measured the kinetics of *putP* substrate specificity mutants. We show that the mutations specifically alter the kinetics of proline transport by *S. typhimurium*.

Materials and Methods

Bacterial Strains

All strains were derived from *S. typhimurium* LT2. The genotypes are shown in Table 1.

Media and Growth Conditions

Nutrient broth (8 g/liter) with NaCl (5 g/liter) was used as a rich medium (NB). Three different minimal media were used: E (Vogel & Bonner, 1956), a medium lacking a carbon source (NCE) (Berkowitz et al., 1968), and a medium lacking both carbon and nitrogen sources (NCN) (Ratzkin, Miklavz & Roth, 1978). Carbon sources were added to minimal media at the following concentrations: 0.2% glucose, 0.6% sodium succinate, 0.3% lactose, and 0.2% melibiose. NCN medium with succinate and 0.2% proline (PSN) was used to test for the ability to grow on proline as a sole nitrogen source. Minimal media were supplemented with a defined amino acid mixture (DAA) when indicated to give the final concentrations listed in Davis et al. (1980). DAA does not cause catabolite repression of the *put* operon and increases the growth rate of cells in minimal medium (Hahn et al., 1988). For transport assays on induced cells, NCE medium containing succinate, proline, and DAA was used. Growth was followed in a Klett-Summerson colorimeter with a green filter. Tetracycline hydrochloride (Tc) was added to rich medium at 25 μg/ml and to minimal media at 10 μg/ml. Kanamycin sulfate (Kn) was added

to rich medium at 50 μg/ml. Chloramphenicol (Cm) was added to rich medium at 20 μg/ml and minimal media at 5 μg/ml. When TTC (2,3,5-triphenyl-2H-tetrazolium chloride) was used as a growth rate indicator (Bochner & Savageau, 1977), it was added to minimal media at 25 μg/ml. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) was dissolved in N,N-dimethylformamide at a concentration of 20 mg/ml and then added to media to yield a final concentration of 20 μg/ml. Bacto agar (Difco Laboratories, Detroit, MI) was added to solid media at 1.5%. Noble agar (Difco) was substituted for Bacto agar in PSN medium to minimize contaminating nitrogen.

Genetic Techniques

Most genetic manipulations used P22 HT105/1 *int*-201, a high frequency generalized transducing phage that cannot form stable lysogens (Schmeiger, 1972). Preparation of phage lysates and transductions were done as previously described (Hahn & Maloy, 1986). Green plates were used in order to isolate phage-free strains (Chan et al., 1972). Sensitivity of strains to phage was verified by cross streaking against H5, a P22 clear plaque mutant (Hahn & Maloy, 1986).

Sensitivity to toxic proline analogs was quantitated by measuring the zone of inhibition after radial streaking (Roth, 1970) on NCE + succinate + TTC plates with proline analogs added to a sterile filter disk placed in the center of each plate. The ability to utilize proline as a sole nitrogen source was assayed in a similar manner by measuring the zone of growth extending from the edge of a sterile disc containing L-proline on NCN + succinate + TTC plates.

Isolation of proP::Tn*10dCm* Insertions

Tn*10dCm* insertions in the *proP* gene were isolated by transducing MS689 (*putP1024::MudJ melB::*Tn*10*) to chloramphenicol resistance (Cm^r) with a phage lysate of a random pool of Tn*10dCm* insertions in LT2 (Elliott & Roth, 1988). Transductants were replica printed onto NB + Tc plates and NCE + melibiose plates to screen for loss of the Tn*10* in *melB*. The Cm^r tetracycline sensitive (Tc^s) Mel$^+$ transductants were screened for enhanced resistance to AZT and DHP relative to MS1209 (*putP1024::*Mu*dJ*) by radial streaking. Phage lysates of putative *proP::*Tn*10dCm* strains were used to transduce MS689 to Cm^r and screened for Tc^s and MelB$^+$. The frequency of Cm^r Tc^s Mel$^-$ transductants was 88%, in agreement with previous reports of linkage between *proP* and *melB* (Menzel & Roth, 1980).

Proline Transport Assays

Cells were grown in NCE + succinate + proline + DAA to mid-log phase, washed twice in 0.85% saline, resuspended in ½ volume NCE at pH 7.0 + 1 mM glucose + 50 μg/ml chloramphenicol, and held on ice until assayed for transport. A 0.2 ml reaction mix containing NCE medium at pH 7.0, 1 mM glucose, 50 μg/ml chloramphenicol, L-[U-^{14}C]proline, and L-proline at the indicated concentrations was added to 7.0-ml plastic mini-vials. Prior to assaying transport, cells (0.25 mg cells dry wt/ml) were swirled on a rotary shaker (100 rpm) for 15 min at room temperature to starve for proline. Then, 0.2 ml of starved cells were rapidly added to the mini-vials containing the reaction mix to initiate the transport assay. At the appropriate time, 5 ml of the stopping buffer (5 mM [N-morpholino] ethanesulfonic acid, 300 mM KCl, 5 mM Tris, and 2 mM $HgCl_2$ at pH 7.0) was rapidly added to the mini-vial to stop the reaction. Uptake of proline was measured over a 15-sec period at 5-sec intervals. The 0-sec time point was obtained by diluting starved cells directly into reaction mix plus stopping buffer. Less than 10 min after transport was halted, transport reaction mixtures were passed through cellulose nitrate filters (0.2-μm pore size; Sartorius) that had been prewet with stopping buffer, then the cells were washed once with 5 ml stopping buffer. All transport assays were carried out at least three times in triplicate. The filters were air-dried, and uptake of proline was determined from the amount of [^{14}C]proline bound to filters by counting in a Beckman 9800 liquid scintillation counter in a full carbon window. A counting efficiency of >96% was obtained when Aquasol (NEN) was used as the scintillation cocktail. All samples were corrected for the low (<100 cpm) ^{14}C background counts due to nonspecific binding of [^{14}C]proline to the filters.

Inhibition of Proline Uptake by Proline Analogs

Proline transport was measured in the presence of inhibitors of proline uptake essentially as described above. Inhibitors were added directly to reaction mixes containing radioactive proline.

Computer Programs

In order to determine the kinetics of proline uptake, we used several kinetic methods: Median ("direct linear plot," Cornish-Bowden & Eisenthal, 1974; Cornish-Bowden, Porter & Trager, 1978), Lineweaver-Burk ($1/v$ *vs.* $1/S$), Hanes (S/v *vs.* S), Eadie-Hofstee (v *vs.* v/S), and Hyper (the best fit of the data to a hyperbola defined by $v = [V \cdot S]/[K_m + S]$; Cleland, 1963). Since the different kinetic methods have shortcomings that do not overlap with one another (Cornish-Bowden, 1979), we ran our results through each kinetic analysis and compared them. The standard deviation from the mean K_m and V_{max} values determined by the different kinetic methods was less than 10% in all cases. The values of K_m and V_{max} cited in the text are derived by the method of Cleland. Computer programs written for kinetic analysis on MS-DOS compatible microcomputers were modified from previously published programs for main-frame computers (Myers, Olson & Maloy, 1990).

Protein Determination

Cells harvested at several points on a growth curve were assayed for total protein concentration by a modification of the Bradford assay (Bio-Rad), and a standard curve of protein concentration *vs.* Klett units was plotted. The amount of protein present in each transport assay was calculated from this plot.

Chemicals

Media and agar were obtained from Difco Laboratories, Detroit, MI. Uniformly labeled L-[^{14}C]proline was obtained from Amersham, Arlington Heights, IL (285 mCi/mmol), or ICN, Irvine, CA (250 mCi/mmol). TTC was obtained from EM Science, Gibbstown, NJ. All other chemicals were obtained from Sigma Chemical, St. Louis, MO.

Table 2. Kinetics of proline transport

Strain	Genotype	K_m[a]	V_{max}[b]
LT2	PutP$^+$ ProP$^+$	2.3 ± 0.3	44.3 ± 1.3
MS700	PutP$^+$ *proP::*Tn*10dCm*	1.8 ± 0.2	47.9 ± 1.3
MS691	*putP1155 proP::*Tn*10dCm*	1.7 ± 0.5	0.6 ± 0.1
MS692	*putP1154 proP::*Tn*10dCm*	215.8 ± 25.6	13.1 ± 0.6
MS695	*putP1160 proP::*Tn*10dCm*	10.5 ± 1.9	1.8 ± 0.1

[a] The mean K_m and SD is expressed as μM proline.
[b] The mean V_{max} and SD is expressed as nmol proline/min^{-1} mg protein^{-1}.

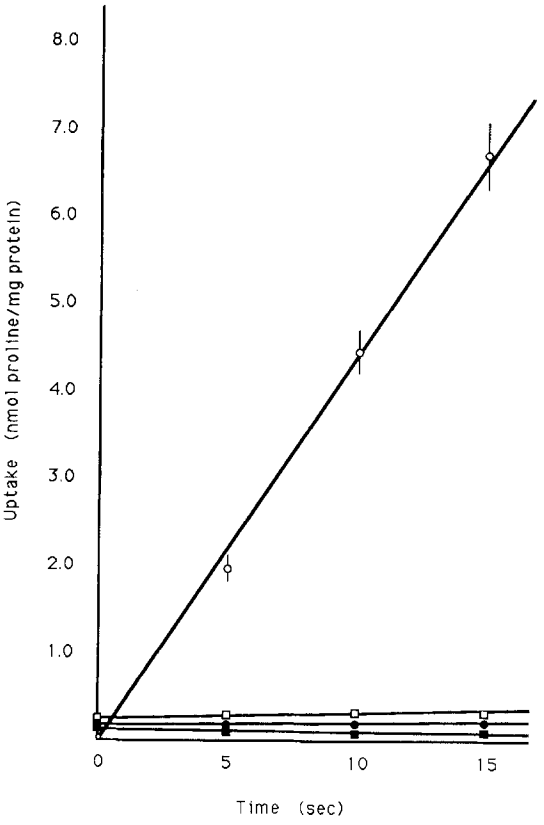

Fig. 2. Transport of L-proline by *S. typhimurium putP* permease. Transport of L-[U-^{14}C]proline was determined as described in Materials and Methods. LT2 (PutP$^+$) induced (○), LT2 uninduced (□); MS1209 (PutP$^-$) induced (●); MS1209 uninduced (■)

Results

Kinetics of Proline Transport by LT2 (PutP$^+$ ProP$^+$)

To determine the kinetics of proline transport in *S. typhimurium*, we sought to measure initial velocities of proline uptake from data taken as quickly as possible after initiation of transport. We initially used a fast filtration technique which halts transport by separating unincorporated proline from cells by simply passing the reaction mix through a filter under vacuum (Chen et al., 1985). By filtration, we could halt transport as early as 15 sec after initiating the reaction with a standard deviation within 10% of the total counts at each time point (*data not shown*). However, we found that significant proline transport occurs in less than 15 sec, and the uptake curve indicates that proline uptake is not "linear" after 15 sec, thus resulting in an underestimate of the initial velocity (*data not shown*). Therefore, we modified a rapid dilution technique developed for determining the kinetics of galactoside transport (Wright & Overath, 1984). The rapid dilution technique can be used to precisely stop proline transport as early as 5 sec after initiating the reaction with an average SD within 5% of the mean (Fig. 2).

The results of proline transport by the wild type *S. typhimurium* LT2 are shown in Fig. 3. Since proline uptake is linear over the first 15 sec, we used this data to estimate the kinetics of proline transport (Table 2). The calculated K_m was 2.3 μM proline, close to the value previously determined for proline transport in *S. typhimurium* and *E. coli* (Wood & Zadworny, 1979; Cairney, Higgens & Booth, 1984). The calculated V_{max} was 44.3 nmol proline/min · mg protein, close to the V_{max} for proline transport in *E. coli* (Wood & Zadworny, 1979) but approximately 10-fold higher than previously reported values in *S. typhimurium* (Cairney et al., 1984). However, this difference may be because the previously published kinetics of proline uptake were derived from *S. typhimurium* cells grown on glucose-containing media, conditions under which expression of the *put* operon is subject to catabolite repression (Ratzkin et al., 1978).

Isolation of *proP::*Tn*10dCm* Mutants

Under most growth conditions, proline transport is primarily mediated by the *putP* gene product in *S. typhimurium* (Ratzkin & Roth, 1978). However, pro-

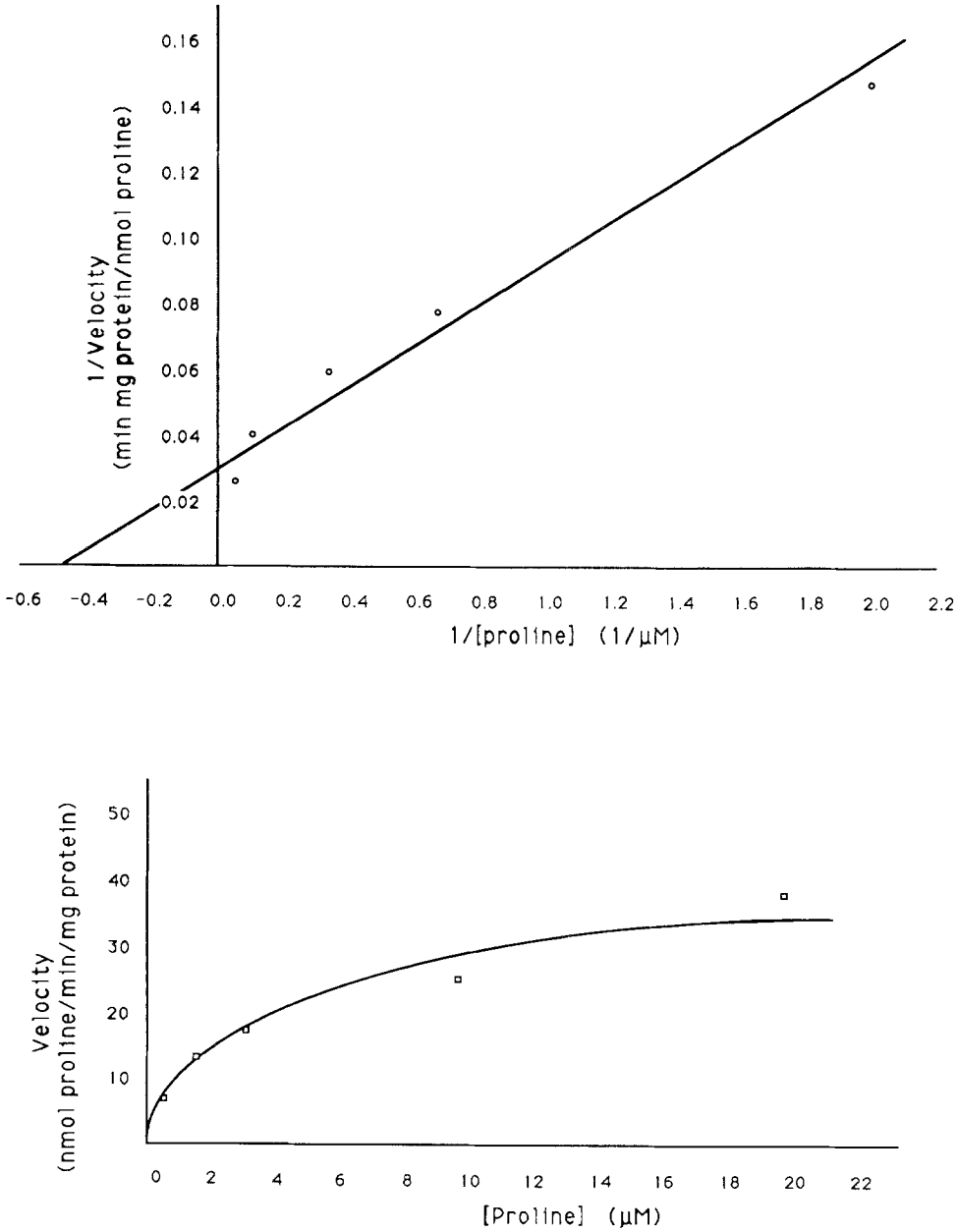

Fig. 3. Kinetics of proline transport by LT2 (PutP$^-$ ProP$^+$). Rate of transport of proline was determined as described in Materials and Methods. A Lineweaver-Burk plot of these data is shown above the Michaelis-Menten plot

line uptake can also be mediated by the *proP* gene product, which is involved in glycine-betaine transport (Cairney, Booth & Higgins, 1985). The K_m for proline by the *proP* permease is over 100-fold higher than the K_m of the *putP* permease (Cairney et al., 1985) and thus would not be expected to contribute greatly to transport at the concentrations of proline used to assay proline permease activity in the wild type. However, we thought it might be possible that some of the *putP* substrate specificity mutants have a lower affinity for proline than the wild type, making the contribution of the *proP* permease to proline transport in these strains significant. In order to make proline transport solely dependent on the *putP* protein we isolated *proP*::Tn*10dCm* insertion mutations by localized transposon mutagenesis.

The *proP* gene maps at 93 min on the *S. typhimurium* genetic map, 88% linked to *melB*, which encodes melibiose permease (Menzel & Roth, 1980). After transducing a *melB*::Tn*10* strain to Cm^r with P22 grown on a random pool of Tn*10dCm* insertions in the *S. typhimurium* chromosome,

Table 3. Phenotypes of strains used in this study

Strain	Phenotype	Pro[a]	DHP[b]	AZT[b]
LT2	PutP$^+$ ProP$^+$	16	24	22
MS1209	PutP$^+$ ProP$^+$	0	4	0
MS690	PutP$^+$ ProP$^-$	0	0	0
MS700	PutP$^+$ ProP$^-$	16	26	23
MS691	*putP1155* ProP$^-$	6	26	0
MS692	*putP1154* ProP$^-$	9	14	0
MS695	*putP1160* ProP$^-$	9	17	0

[a] The ability to use proline as a sole nitrogen source was quantitated by radial streaking from a filter disc impregnated with 8 mg L-proline and measuring the zone of growth extending from the edge of the disc.

[b] Sensitivity to toxic proline analogs was quantitated by radial streaking from a filter disc impregnated with either 400 μg DHP or 500 μg AZT and measuring the zone of growth inhibition extending from the edge of the disc.

*proP::*Tn*10dCm* insertion mutants were enriched by selecting for Mel$^+$ Cm^r colonies. The *proP* permease can transport DHP in addition to proline. However, since the *putP* permease can transport DHP also, this ProP$^-$ phenotype can only be observed in a *putP* background. Therefore, we looked for *proP::*Tn*10dCm* insertions in MS689 (*putP1024::*Mu*dJ melB363::*Tn*10*) by screening Cm^r Tc^s Mel$^+$ transductants for enhanced resistance to high concentrations of the toxic proline analog DHP.

Four strains were isolated that showed enhanced resistance to DHP relative to MS689. P22 lysates of these strains were used to transduce MS689 to Cm^r and coinheritance of Tc^s, Mel$^+$ and enhanced DHP^r was scored. DHP^r was 100% linked to Cm^r (100 colonies scored) while Tc^s Mel$^+$ was 88% linked to Cm^r (ca. 1000 colonies scored) for each of the strains. One of the transductants, MS690, was used as a donor in all further studies with *proP::*Tn*10dCm*.

Kinetics of Proline Transport in PutP$^+$ ProP$^-$ Cells

In order to measure proline transport by the *putP* gene product alone, we transduced LT2 to Cm^r with phage grown on MS690 (*proP::*Tn*10dCm*) and we determined the kinetics of proline transport in this strain (MS700). The results are shown in Fig. 4*A* and summarized in Table 2. The K_m for proline in the *proP* mutant was 1.8 μM, very similar to the value obtained for LT2 (2.3 μM) and the V_{max} was 47.9 nmol proline/min · mg protein, similar to the V_{max} of LT2 (44.3 nmol/min · mg). These results confirm previous studies, indicating that the *proP* gene product makes a negligible contribution to proline uptake by PutP$^+$ cells at the proline concentrations used (Anderson, Menzel & Wood, 1980; Grothe et al., 1986).

Comparison of PutP$^+$ PutP$^-$, and *putP* Substrate Specificity Mutants in the Absence of the *proP* Permease

In order to assess the effect of the *proP::*Tn*10dCm* mutation on the phenotype of *putP* mutants, we examined their ability to use proline and their sensitivity to proline analogs by the radial streak technique (Table 3). MS1209 (PutP$^-$ ProP$^+$) is unable to use proline as a sole nitrogen source and is resistant to toxic proline analogs. MS690 (PutP$^-$ ProP$^-$) is more resistant to the analog DHP than is MS1209. MS700 (PutP$^+$ ProP$^-$) resembles the wild type, LT2 (PutP$^+$ ProP$^+$), indicating that the *putP* gene is epistatic to *proP*, consistent with our results for proline transport by LT2 and MS700. Growth of three substrate specificity mutants (MS691, MS692 and MS695) on proline was decreased compared to the isogenic PutP$^+$ control strain, MS700. The substrate specificity mutants also showed different analog resistance patterns than MS700. For example, MS691 was more resistant to the toxic proline analog AZT than MS700, yet remained as sensitive to a second toxic analog, DHP, as MS700; MS692 showed increased resistance to all proline analogs tested as did MS695. If proline analogs are alternative substrates of proline permease, all of the radial streak phenotypes of *putP* substrate specificity mutants can be explained by defects in proline permease that alter substrate binding or translocation. This interpretation rests on the demonstration that proline analogs are proline permease substrates.

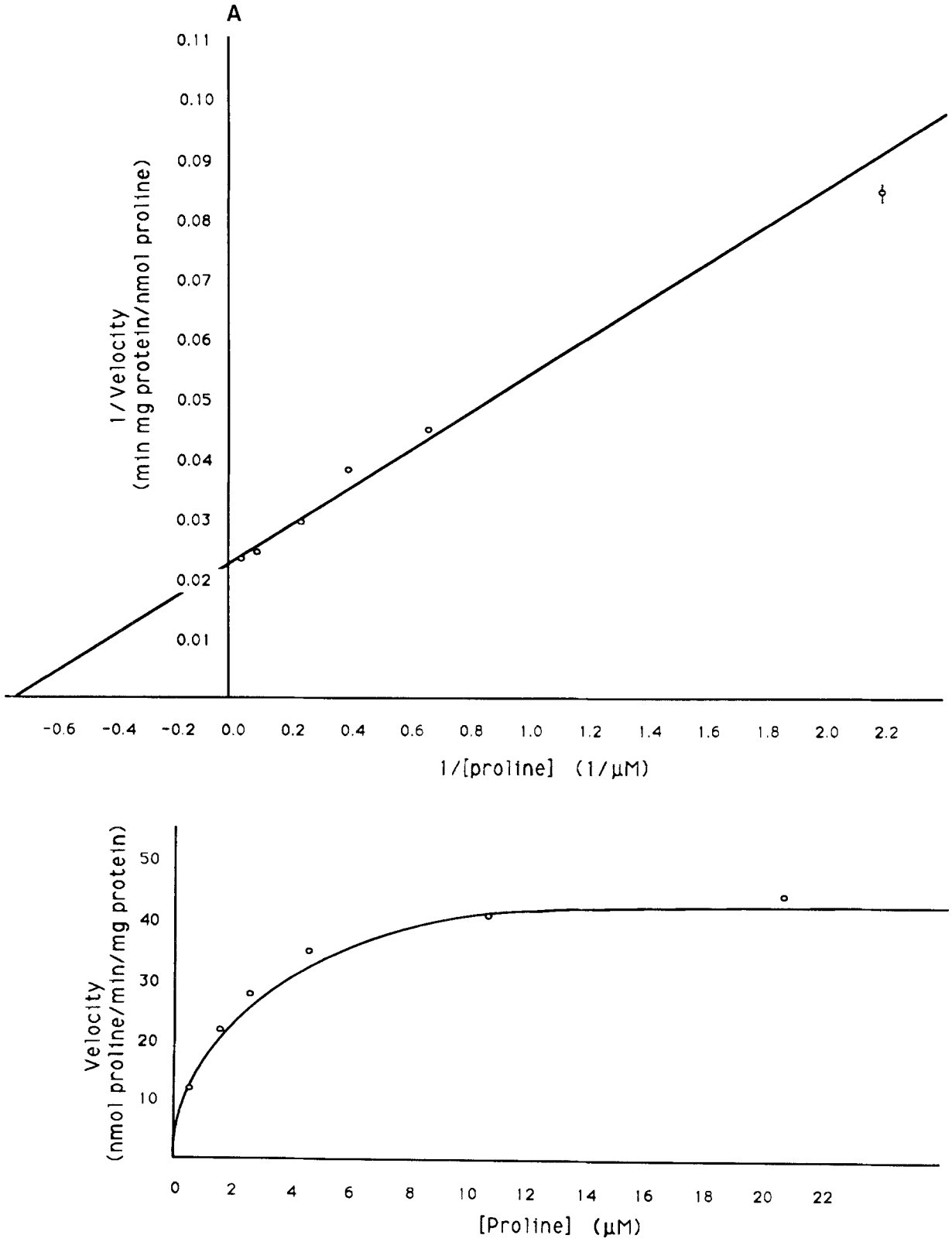

Fig. 4. Kinetics of proline transport by PutP$^+$ and *putP* substrate specificity mutant permeases in ProP$^-$ cells. The rate of transport by MS700 (PutP$^-$ ProP$^-$; *A*), MS691 (*putP1155* ProP$^-$; *B*), and MS692 (*putP1154* ProP$^-$; *C*), and MS695 (*put1160* ProP$^-$; *D*) was determined as described in Materials and Methods. Lineweaver-Burk plots of these data are shown above each Michaelis-Menten plot

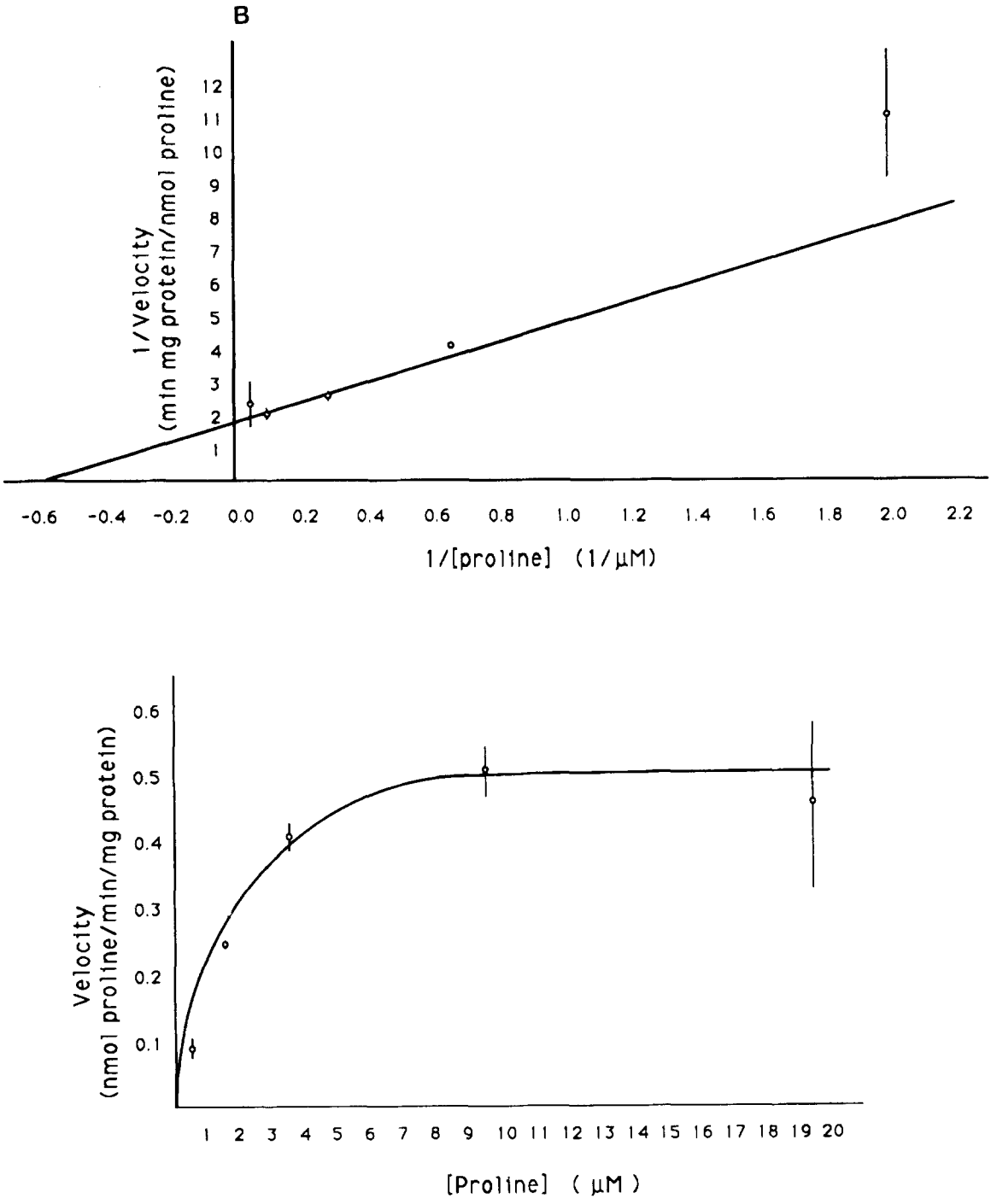

Fig. 4—Continued

Inhibition of Proline Transport by Proline Analogs is Competitive

Substrate specificity mutants were isolated based on altered discrimination of proline permease substrates, proline and toxic proline analogs. In order to determine if proline analogs are alternate substrates of proline permease, we wanted to measure transport of proline analogs. Radioactive proline analogs are not commercially available, so we studied the inhibition of [^{14}C]proline transport by unlabeled analogs. First the inhibition of [^{14}C]proline uptake by unlabeled L-proline was measured in MS700. As expected, inhibition of proline uptake by proline was

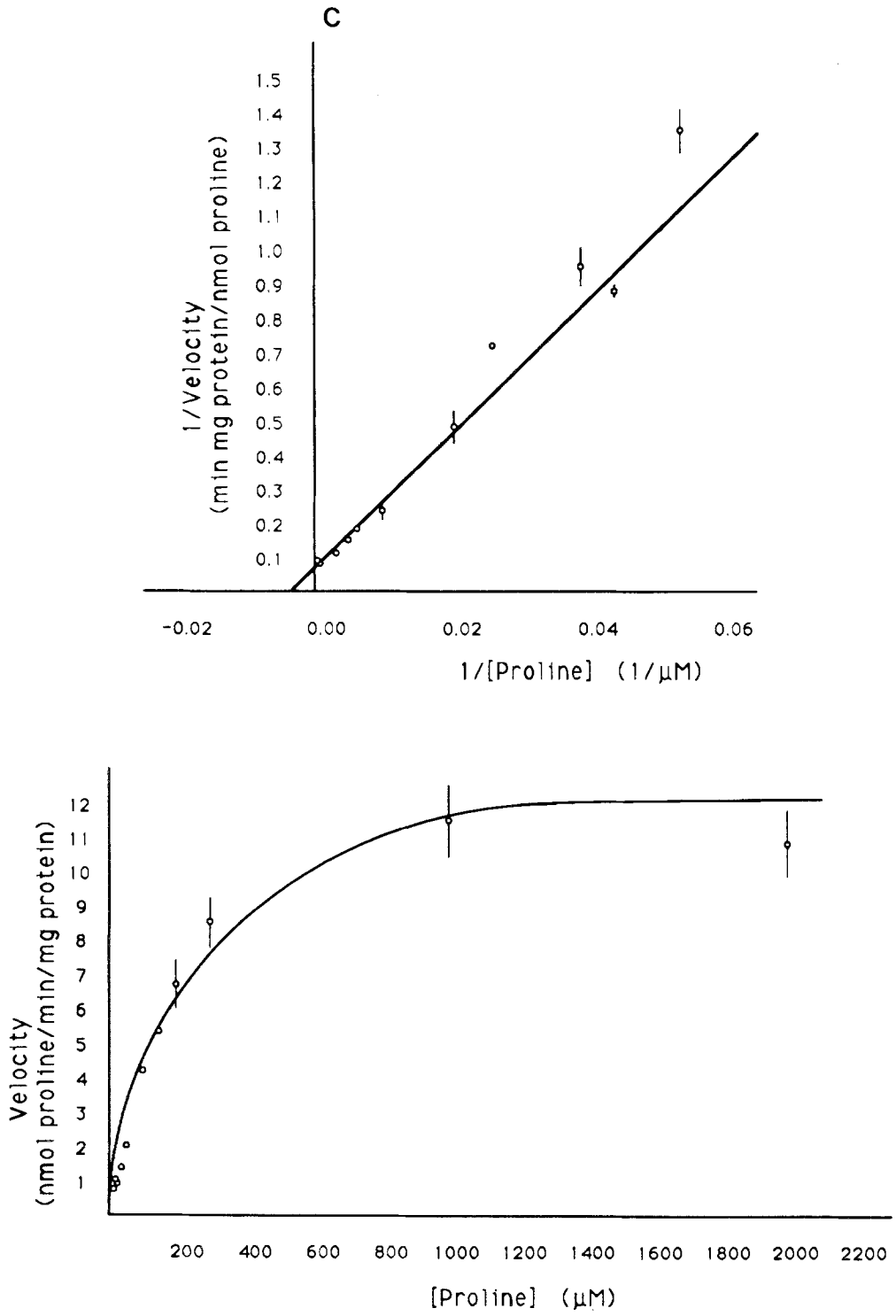

Fig. 4—Continued

competitive (Fig. 5*A*). The K_i obtained for inhibition by proline was 1.3 μM, nearly identical to the K_m for proline transport.

Next, we examined inhibition of proline uptake by two proline analogs, DHP and AZT. Previous studies of the substrate specificity of proline permease suggested that many proline analogs inhibit proline uptake, and that AZT and DHP can exchange with intracellular [^{14}C]proline (Rowland & Tristam, 1975). This data suggest that AZT and DHP are

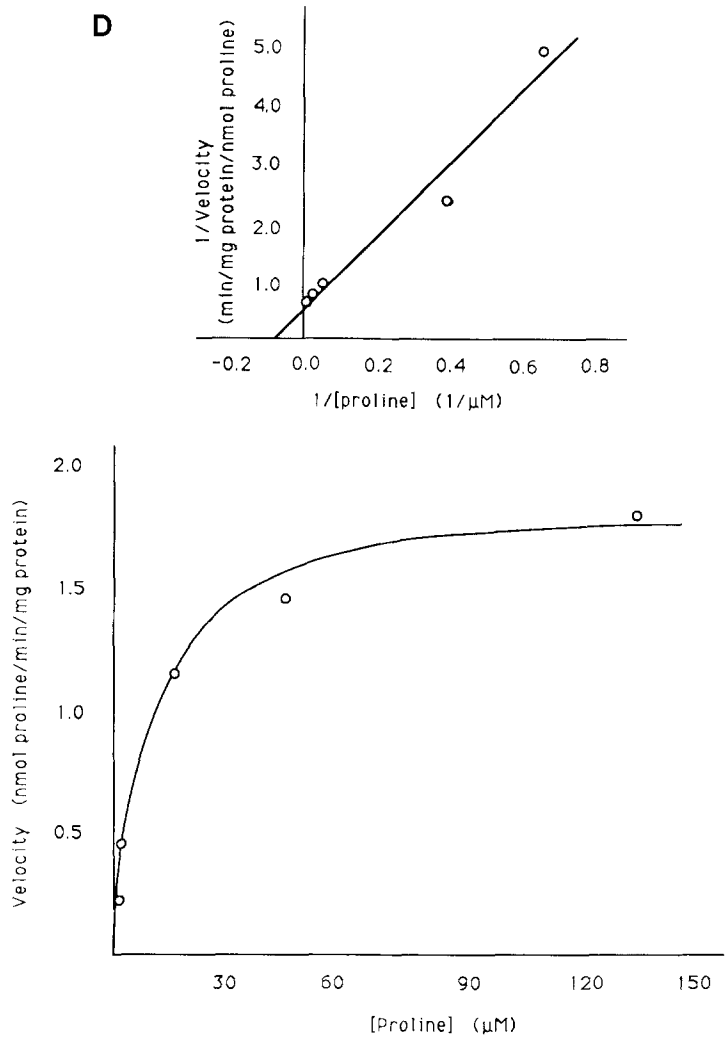

Fig. 4—Continued

transported by proline permease. If so, AZT and DHP should competitively inhibit proline transport. Thus, the K_i values should equal the K_m for AZT and DHP transport just as the K_i for inhibition of proline transport by proline was the same as the K_m for proline transport (Cornish-Bowden, 1979). In our assay, DHP competitively inhibited proline uptake (Fig. 5*B*). Since DHP was a 50 : 50 mixture of the D- and L-enantiomers, we calculated the K_i to be 9 μM (assuming only the L-enantiomer is active; Rowland & Tristam, 1975). Likewise, AZT acted as a competitive inhibitor of proline uptake with a K_i of 125 μM (Fig. 5*C*). These results are consistent with observations that lower concentrations of DHP are needed to inhibit cell growth than AZT (Table 3).

Kinetics of Proline Transport by *putP* Substrate Specificity Mutants

We determined the kinetics of proline transport by several substrate specificity mutants that were transduced to *proP::*Tn*10dCm*. Each one of the *putP* substrate specificity mutants was originally isolated as being "leaky" for growth on proline as a sole nitrogen source ($PSN^{+/-}$, but shows different analog sensitivity patterns (Table 3). MS691 (*putP1055 zcc-7::*Tn*10 proP::*Tn*10dCm*), exhibits decreased transport of proline and AZT, yet appears to transport DHP as well as the wild type (Table 3). The results of proline transport by MS691 are shown in Fig. 4*B* and summarized in Table 2. The K_m for proline was 1.7 μM, essentially identical to that of the isogenic $PutP^+$ strain; however, the V_{max} was 0.6 nmol proline/min · mg protein, roughly 80-fold lower than that of the $PutP^+$ derivative. For this reason, we refer to *putP1155* as a V_{max} mutation.

Another mutant, MS692 (*putP1154 zcc-7::*Tn*10 proP::*Tn*10dCm*), exhibited decreased transport of all proline permease substrates, as determined by radial streak tests (Table 3). Using concentrations of proline sufficient for determining the kinetics of MS700 and MS691, we obtained negative values for K_m and V_{max} (*data not shown*), suggesting that we

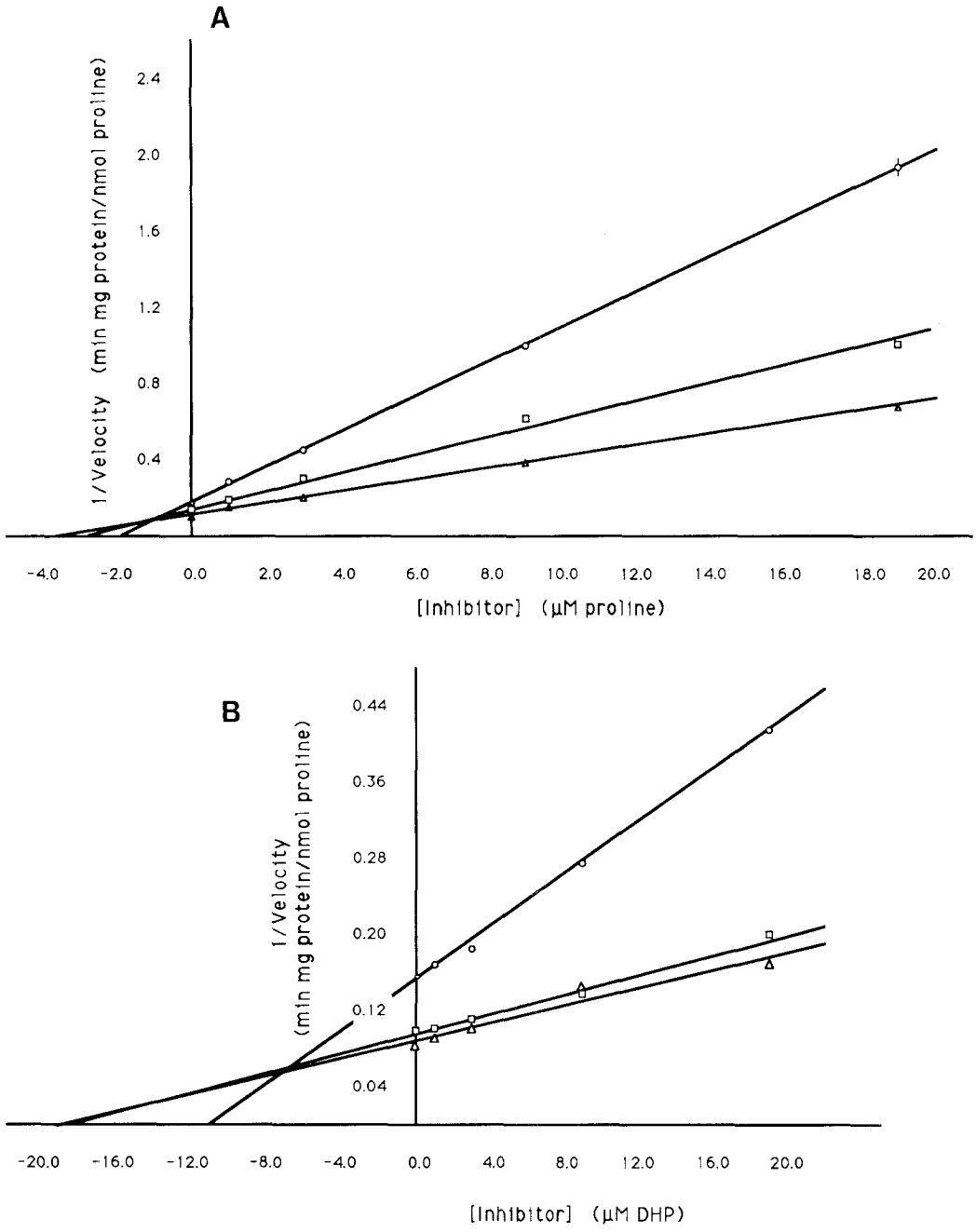

Fig. 5. Inhibition of proline transport by MS700 (PutP$^-$ ProP$^-$). Rate of transport of L-[U-^{14}C]proline in the absence of L-proline (*A*; [^{14}C]proline concentrations: ○ = 0.5 μM, □ = 1.0 μM, △ = 1.5 μM), DL-DHP (*B*; [^{14}C]proline concentrations: ○ = 0.25 μM, □ = 0.5 μM, △ = 0.75 μM), or L-AZT (*C*; [^{14}C]proline concentrations: ○ = 0.25 μM, = 0.75 μM, △ = 1.5 μM) was determined as described in Materials and Methods. Dixon plots of these data are shown

were assaying proline transport well below the K_m value for the mutant proline permease (Cornish-Bowden, 1979). When transport was carried out at much higher proline concentrations, we found that the K_m for proline transport was 216 μM, approximately 120 times that of the isogenic PutP$^+$ strain, while V_{max} was only decreased fourfold (13 nmol proline/min · mg protein) (*see* Fig. 4*C* and Table 2). Therefore, we refer to *putP1154* as a K_m mutation.

The mutant MS695 (*putP1160 zcc-7::*Tn*10 propP::*Tn*10dCm*) resembles the K_m mutant in radial streak tests (Table 3) but exhibits lower affinity and decreased rates of proline uptake relative to the isogenic control (MS700). The K_m for proline transport by MS695 was 10.5 μM, approximately sixfold higher than that of MS700, and the V_{max} was 1.8 nmol proline/min mg protein, almost 27 times lower than MS700. Therefore, *putP1160* has a significant

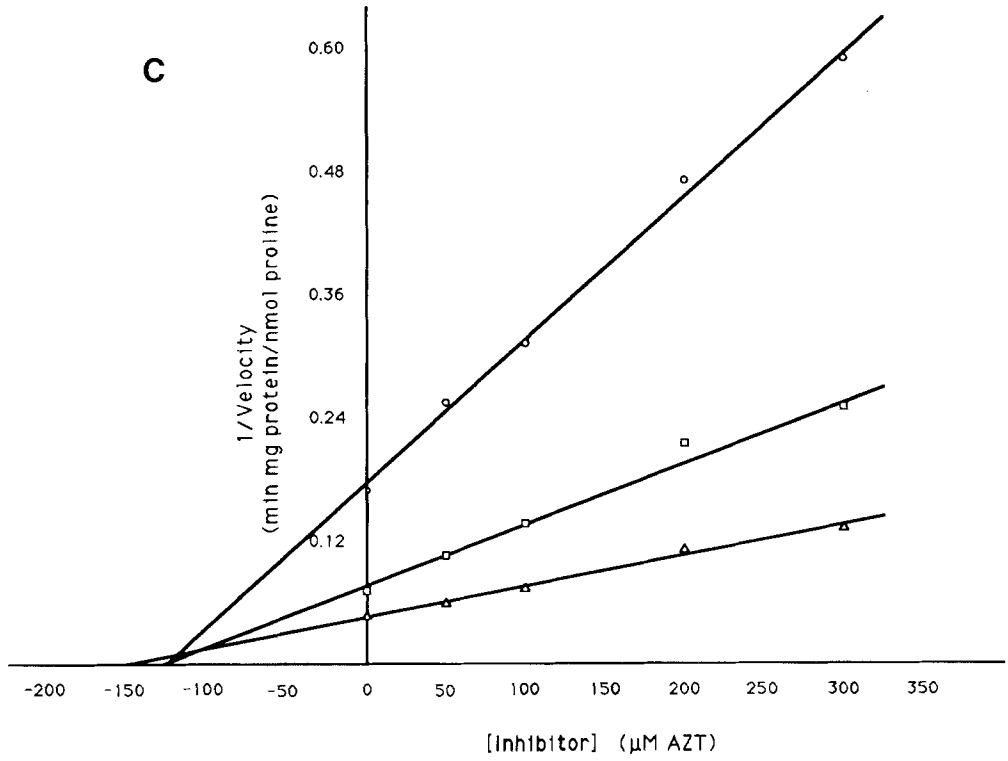

Fig. 5—Continued

effect on both V_{max} and K_m. Preliminary results indicate that mutations which affect both proline binding and translocation are among the most frequently isolated as *putP* substrate specificity mutants (*data not shown*).

V_{max} Effects are Caused by Transport Defects

The decrease in V_{max} seen in the substrate specificity mutants could result from four factors: (*i*) decreased transcription of the *putP* gene, (*ii*) decreased translation of *putP* message, (*iii*) decreased activity of the *putP* protein, and (*iv*) increased degradation of the mutant *putP* proteins. We tested each of these possibilities by making *putP::lacZ* operon fusions in *trans* to substrate specificity mutations to measure transcriptional effects and *putP-lacZ* gene fusions in *cis* to substrate specificity alleles to measure translation, membrane localization and degradation of mutant proline permeases. Substrate specificity mutations do not effect translation, localization or turnover of proline permease-B-galactosidase hybrid proteins (*data not shown*). The mutant permeases decrease uptake of the inducer (proline), but the effect on transcription rates is modest (1.5- to 3-fold decreases) indicating that decreases in the V_{max} are primarily due to catalytic defects of the mutants.

Discussion

A large number of *putP* substrate specificity mutations have been previously isolated in order to define the active site of proline permease. If *putP* substrate specificity mutations directly alter the active site of proline permease, they should be recovered less frequently than simple structural mutations (such as null mutations). In addition, active site mutations should map to a limited number of regions in the gene, whereas null mutations are found throughout the structural gene. As predicted, mutations that alter the substrate or cation specificity of proline permease are rare, and cluster in small regions of the *putP* gene (Dila & Maloy, 1986; Myers & Maloy, 1988). A final prediction is that *putP* active site mutations should specifically alter the kinetics of proline transport by proline permease. Therefore we determined the kinetics of proline transport by *putP* substrate specificity mutants.

In order to determine the kinetics of proline transport, we developed an assay system that allowed us to precisely measure proline uptake as soon as 5 sec after initiating proline transport. In order to eliminate contributions to proline uptake by the *proP* permease, we isolated transposon insertion mutants of *proP*. We then measured the kinetics of proline transport by the wild type and mutant proline permease in isogenic *proP::*Tn*10dCm* backgrounds.

The kinetcis of proline transport by the wild type proline permease was similar to previously published values (Wood & Zadworny, 1979; Cairney et al., 1984) with a specificity constant (V_{max}/K_m) of 26.6. In contrast, a *putP*::Mud*J* mutant fails to transport proline (Fig. 2). As predicted, *putP* substrate specificity mutations specifically alter the kinetics of proline transport. One mutation *putP1154* increases the K_m over 120-fold without greatly affecting the V_{max} ($V_{max}/K_m = 0.06$). This K_m mutant may be specifically defective in substrate recognition, thereby identifying an amino acid critical for substrate binding. Another mutation, *putP1155*, decreased the V_{max} 80-fold without changing the K_m ($V_{max}/K_m = 0.4$). This V_{max} mutant appears to alter substrate translocation without affecting the substrate binding site. A third substrate specificity mutation (*putP1160*) both decreased the V_{max} and increased the K_m for proline uptake ($V_{max}/K_m = 0.2$). Thus, by specifically affecting the kinetics of proline transport, *putP* substrate specificity mutations satisfy the third prediction of mutations that alter the active site of proline permease.

Mutations that decrease the rate of proline uptake may decrease the V_{max} of proline transport in two ways: by lowering the rate of catalysis (K_{cat}) directly *and* by decreasing the number of mutant permeases in the membrane due to inducer exclusion (K_{cat} · number of permeases = V_{max}). However, the decrease in *put* transcription due to inducer exclusion made only a minor contribution to decreased proline permease activity. Therefore, V_{max} defects in the mutant permeases are primarily due to catalytic defects.

In conclusion, *putP* substrate specificity mutants act like *bona fide* active site mutants. These results indicate that screening for substrate specificity mutations induced by localized random mutagenesis is a powerful approach for dissecting the active site of enzymes which have more than one substrate.

We thank J. Keith Wright for useful comments and stimulating discussions. John Roth and Tom Elliott generously provided strains. This work was supported by NIH grant GM39096 to S.M.

References

Anderson, R., Menzel, R., Wood, J. 1980. Biochemistry and regulation of a second L-proline transport system in *Salmonella typhimurium*. *J. Bacteriol.* **141:**1071–1076

Berkowitz, D., Huston, J., Whitfield, H., Roth, J., Ames, B. 1968. Procedure for identifying nonsense mutations. *J. Bacteriol.* **96:**215–220

Bochner, B., Savageau, M. 1977. Generalized indicator plate for genetic, metabolic, and taxonomic studies with microorganisms. *Appl. Environ. Microbiol.* **33:**434–444

Botfield, M., Wilson, T. 1988. Mutations that simultaneously alter both sugar and cation specificity of the melibiose carrier of *Escherichia coli*. *J. Biol. Chem.* **263:**12909–12915

Brooker, R., Wilson, T.H. 1985. Isolation and nucleotide sequencing of lactose carrier mutants that transport maltose. *Proc. Natl. Acad. Sci. USA* **82:**3959–3963

Cairney, J., Booth, I., Higgins, C. 1985. *Salmonella typhimurium proP* gene encodes a transport system for the osmoprotectant betaine. *J. Bacteriol.* **164:**1218–1223

Cairney, J., Higgins, C., Booth, I. 1984. Proline uptake through the major transport system of *Salmonella typhimurium* is coupled to sodium ions. *J. Bacteriol.* **160:**22–27

Chan, R., Botstein, D., Watanabe, T., Obaya, Y. 1972. Specialized transduction of teracycline resistance by phage P22 in *Salmonella typhimurium*. *Virology* **50:**833–898

Chen, C.-C., Tsuchiya, T., Yamane, Y., Wood, J.M., Wilson, T.H. 1985. $Na^+(Li^+)$-proline cotransport in *Escherichia coli*. *J. Membrane Biol.* **84:**157–164

Cleland, W. 1963. Computer programmes for processing enzyme kinetic data. *Nature* **198:**463

Cornish-Bowden, A. 1979. Fundamentals of Enzyme Kinetics. Butterworths, London

Cornish-Bowden, A., Eisenthal, R. 1974. Statistical considerations in the estimation of kinetic parameters by the direct linear plot. *Biochem. J.* **139:**721–730

Cornish-Bowden, A., Porter, W., Trager, W., Trager, W. 1978. Evaluation of distribution-free confidence limits for enzyme kinetic parameters. *J. Theor. Biol.* **74:**163–175

Davis, R., Botstein, D., Roth, J. 1980. Advanced Bacterial Genetics. Cold Spring Harbor Lab., Cold Spring Harbor (NY)

Dila, D., Maloy, S. 1986. Proline transport in *Salmonella typhimurium: putP* permease mutants with altered substrate specificity. *J. Bacteriol.* **168:**590–594

Elliott, T., Roth, J. 1988. Tn*10d-Cam:* Transposase-defective Tn*10* specifying chloramphenicol resistance. *Mol. Gen. Genet.* **213:**332–338

Fox, C., Kennedy, E. 1965. Specific labelling and partial purification of the M protein, a component of the B-galactoside transport protein of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **54:**891–899

Grothe, S., Krogsrud, R., McClellan, D., Milner, J., Wood, J. 1986. Proline transport and osmotic stress response in *Escherichia coli* K12. *J. Bacteriol.* **166:**253–259

Hahn, D., Maloy, S. 1986. Isolation and characterization of *cis*-dominant regulatory mutations in the *put* operon of *Salmonella typhimurium*. *Genetics* **114:**687–703

Hahn, D., Myers, R., Kent, C., Maloy, S. 1988. Regulation of proline utilization in *Salmonella typhimurium:* Molecular characterization of the *put* operon and DNA sequence of the *put* control region. *Mol. Gen. Genet.* **213:**125–133

Hong, J., Ames, B. 1972. Localized mutagenesis of any specific small region of the bacterial chromosome. *Proc. Natl. Acad. Sci. USA* **68:**3158–3162

Kaback, H. 1987. Use of site-directed mutagenesis to study the mechanism of a membrane transport protein. *Biochemistry* **26:**2071–2076

Maloy, S. 1987. The proline utilization operon. *In: Escherichia coli* and *Salmonella typhimurium:* Cellular and Molecular Biology. F.C. Neidhardt, editor. pp. 1513–159. American Society for Microbiology, Washington, D.C.

Menzel, R., Roth, J. 1980. Identification and mapping of a second proline permease in *Salmonella typhimurium*. *J. Bacteriol.* **141:**1064–1070

Mieschendahl, M., Buchel, D., Bocklage, H., Mueller-Hill, B.

1981. Mutations in the *lacY* gene of *Escherichia coli* define functional organization of lactose permease. *Proc. Natl. Acad. Sci. USA* **78:**7652–7656

Myers, R., Dila, D., Maloy, S., 1987. Mutations that alter the substrate specificity of proline permease in *Salmonella typhimurium*. *In:* Membrane Proteins. S. Goheen, editor. pp. 471–481. Bio-Rad Laboratories, Richmond (CA)

Myers, R., Maloy, S. 1988. Mutations of *putP* that alter the lithium sensitivity of *Salmonella typhimurium*. *Mol. Microbiol.* **2:**749–755

Myers, R., Olson, S., Maloy, S. 1990. Computer programs for the rapid determination of enzyme kinetics on MS-DOS compatible microcomputers. *Comput. Appl. Biol. Sci.* **6:**63–65

Niiya, S., Yamasaki, K., Wilson, T.H., Tsuchiya, T. 1982. Altered cation coupling to melibiose transport in mutants of *Escherichia coli*. *J. Biol. Chem.* **257:**8902–8906

Ratzkin, B., Miklavz, G., Roth, J. 1978. Regulation of the major proline permease gene of *Salmonella typhimurium*. *J. Bacteriol.* **133:**737–743

Ratzkin, B., Roth, J. 1978. Cluster of genes controlling proline degradation in *Salmonella typhimurium*. *J. Bacteriol.* **133:**744–754

Roth, J. 1970. Genetic techniques in studies of bacterial metabolism. *Methods Enzymol.* **17A:**3–35

Rowland, I., Tristam, H. 1975. Specificity of the *Escherichia coli* proline transport system. *J. Bacteriol.* **123:**871–877

Sanderson, K., Roth, J. 1988. Linkage map of *Salmonella typhiminium,* Edition VII. *Microbiol. Rev.* **52:**485–452

Schmeiger, H. 1972. Phage P22 mutants with increased or decreased transduction abilities. *Mol. Gen. Genet.* **119:**75–88

Shiota, S., Yamane, Y., Futai, M., Tsuchiya, T. 1985. *Escherichia coli* mutants possessing a Li^+-resistant melibiose carrier. *J. Bacteriol.* **162:**106–109

Trumble, W., Viitmen, P., Sarka, H., Poonian, M., Kaback, H. 1984. Site-directed mutagenesis of cys_{148} in the *lac* carrier protein of *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **119:**860–867

Viitmen, P., Menick, D., Sarka, H., Trumble, W., Kaback, H. 1985. Site-directed mutagenesis of cysteine-148 in the *lac* permease of *Escherichia coli:* Effect on transport, binding and sulfhydral inactivation. *Biochemistry* **24:**7628–7635

Vogel, H., Bonner, D. 1956. Acetylornithinase of *Escherichia coli*. Partial purification and some properties. *J. Biol. Chem.* **218:**97–106

Wilson, D. 1978. Cellular transport mechanisms. *Annu. Rev. Biochem.* **47:**933–965

Wood, J., Zadworny, D. 1979. Characterization of an inducible porter required for L-proline catabolism by *Escherichia coli* K12. *Can. J. Biochem.* **57:**1191–1199

Wright, J.K., Overath, P. 1984. Purification of the lactose:H^+ carrier of *Escherichia coli* and characterization of galactoside binding and transport. *Eur. J. Biochem.* **138:**497–508

Received 13 November 1990