Articles

Substrate and Inhibitor-Induced Conformational Changes in the Structurally Related Enzymes UDP-*N*-Acetylglucosamine Enolpyruvyl Transferase (MurA) and 5-Enolpyruvylshikimate 3-Phosphate Synthase (EPSPS)†

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ABSTRACT: UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) and 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) have both a unique three-dimensional topology and overall reaction mechanism in common. In the case of MurA, the substrate-free, unliganded protein exhibits an "open" conformation. Upon binding of substrates, the protein forms a much more tightly packed so-called "closed" form following an induced fit mechanism. In this closed form, the substrates are properly positioned for catalysis. On the basis of the structural and mechanistic similarities of MurA and EPSPS, a similar conformational change is likely to occur in EPSPS to generate a catalytically competent active site. However, there is currently little experimental evidence available to support the occurrence of such a conformational change in EPSPS. Using limited tryptic digestion of MurA,[1] it could be shown that formation of the "closed" conformation of MurA is accompanied by a marked increase of stability toward proteolytic degradation. Formation of the closed conformation was achieved by addition of either an excess of both substrates or the sugar nucleotide substrate in conjunction with the antibiotic fosfomycin. Analysis of the MurA tryptic fragments by MALDI-TOF mass spectrometry demonstrates that the protection of the protein in either case is caused by (1) a specific shielding of regions thereby becoming less accessible as a result of the conformational change, and (2) an unspecific overall protection of the whole protein due to an apparently reduced flexibility of the peptide backbone in the binary and ternary complexes. The establishment of methods to describe the effects of tryptic digestion on MurA under various conditions was then extended to EPSPS. Although EPSPS was found to be much more stable toward proteolysis than MurA, the presence of shikimate 3-phosphate (S3P) and the inhibitor glyphosate led to a pronounced suppression of proteolytic degradation. When unliganded EPSPS was treated with trypsin, three of the peptide fragments obtained could be identified by mass spectrometry. Two of these are located in a region corresponding to the "catalytic" loop in MurA which participates in the conformational change. This indicates a conformational change in EPSPS, similar to the one observed in MurA, leading to the protection mentioned above. Corroborating evidence was obtained using a conformational sensitive monoclonal antibody against EPSPS which showed a 20-fold reduced affinity toward the protein complexed with S3P and glyphosate as compared to the unliganded enzyme.

UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) and 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) are the only reported enzymes which catalyze a C(2)−O bond cleavage of phosphoenolpyruvate (PEP) accompanied by an enolether transfer to the hydroxyl group of the substrate (ref *1*; Scheme 1). Moreover, they have a unique structural topology, referred to as an "inside-out α/β-barrel" or "mushroom button" (*2*, *3*), in common.

Both enzymatic reactions are important steps in major biosynthetic pathways: MurA catalyzes the first committed step in the biosynthesis of murein, an essential cell wall component of eubacteria (*4*), while EPSPS catalyzes the sixth step in the shikimate pathway, the major source of aromatic compounds, such as aromatic amino acids, siderophores, and lignins, in microorganisms, fungi, and plants (*5*). For this reason, both enzymes are suitable targets for the development

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[1] Abbreviations: EPSPS, 5-enolpyruvylshikimate 3-phosphate synthase; Fm, fosfomycin (1*R*,2*S*)-1,2-epoxypropylphosphonic acid); Glp, glyphosate [*N*-(phosphonomethyl) glycine]; MALDI-TOF, matrix-assisted laser desorption/ionization−time-of-flight; MCA 1, monoclonal antibody 1; MurA, UDP-*N*-acetylglucosamine enolpyruvyl transferase; PEP, phosphoenolpyruvate; S3P, shikimate 3-phosphate; UDPNAG, UDP-*N*-acetylglucosamine.

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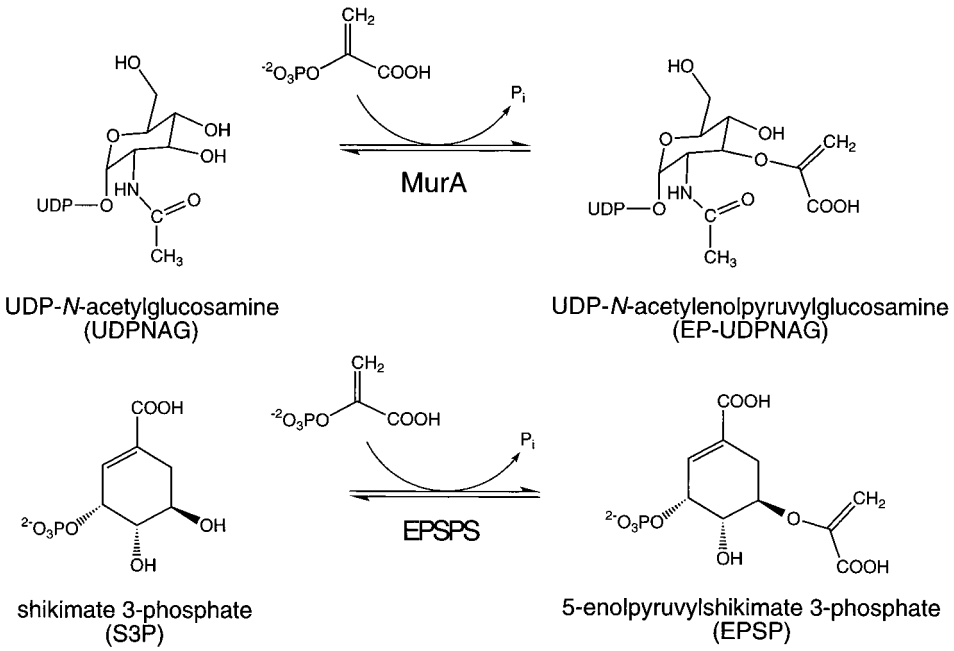
Scheme 1



or improvement of herbicides and antibiotics. In fact, EPSPS is the target of the herbicide glyphosate (*6*), while MurA is inhibited by the antibiotic fosfomycin (*7*). Despite the similarity of the enzymes on the structural and mechanistic level, the mode of action of these inhibitors is quite different. Fosfomycin is covalently attached to MurA by nucleophilic attack of the thiol group of cysteine 115 (*8*, *9*), which is located in a flexible loop of MurA (*3*, *10*). Glyphosate, on the other hand, is a noncovalently bound inhibitor of EPSPS which is thought to act at an allosteric site (*11*).

For EPSPS, as well as for MurA, a random mechanism of substrate binding and a strong substrate synergism or induced fit mechanism, respectively, have been proposed recently (*12*−*14*).

Structural studies of MurA revealed the existence of two distinct conformations: in the absence of substrates, the protein exists in an "open" form and undergoes a marked conformational change toward a more compact, so-called closed form, in the presence of the sugar nucleotide substrate (UDPNAG) and the antibiotic fosfomycin (*3*, *10*). A closed structure was also reported recently for the C115A mutant protein of *Escherichia coli* MurA complexed with the fluorinated analogue of the tetrahedral reaction intermediate (*15*). Conformational changes in MurA upon ligand binding have also been shown by small-angle X-ray scattering studies (*14*). On the other hand, the only 3D-structure (to 3 Å resolution) currently available of EPSPS is that of the ligand free open form (*2*), but a similar substrate-induced conformational change, like that of MurA, has been proposed for EPSPS. Such a conformational change was indirectly derived from chloroplast import studies (*16*), isothermal calorimetry (*13*), fluorescence quench (*17*) and NMR experiments (*18*, *19*). None of these methods has until now been applied to MurA, nor has any method ever been applied to both enzymes in parallel by one and the same laboratory. Therefore, one objective of the present work was to provide further evidence for similar conformational changes in MurA and EPSPS upon substrate and/or inhibitor binding by investigating both enzymes employing a single method.

In this work, it is shown that the substrate-induced conformational changes in MurA result in protection against tryptic digestion of the protein. The extent of protection of MurA depends on the relative concentration of the two substrates UDPNAG and PEP as well as the presence of the antibiotic fosfomycin. This protection of MurA toward proteolysis was characterized employing SDS−PAGE, enzyme activity assays, and MALDI-TOF mass spectrometry. Since the observed protection correlates with the formation of the closed form of MurA, we exploited these methods in a comparative investigation of EPSPS. It was found that EPSPS is substantially stabilized against tryptic digestion in the presence of its substrate shikimate-3-phosphate (S3P) and the herbicide glyphosate. This result suggests that EPSPS undergoes a similar conformational change to that of MurA, i.e., that binding of S3P and glyphosate results in the formation of a closed form of the protein. Results obtained with a monoclonal antibody raised against the free, uncomplexed form of EPSPS support this interpretation. This antibody showed a strongly reduced affinity toward EPSPS complexed with S3P and glyphosate. Besides establishing the similarity of the protection against tryptic digestion, this comparative study of MurA and EPSPS also reveals that, despite the distinct mode of action of fosfomycin and glyphosate, their interaction with the protein results in a much tighter and rigid structure restricting the flexibility of the enzyme required for the exchange of substrate and product molecules. This impediment of the dynamic features of an enzyme appears to be an important aspect of the inhibition event which could be exploited in the search for new enzyme inactivators.

MATERIALS AND METHODS

Materials. All chemicals were of the highest available grade from Fluka (Buchs, CH); the potassium salt of PEP was used throughout the experiments. UDPNAG and fosfomycin were from Sigma, shikimic acid was from Roth (Karlsruhe, Germany), and dihydroxy benzoic acid was from Aldrich. Shikimate-3-phosphate was produced as described

by Stepanek (*20*). Glyphosate (>99%) was a gift from Monsanto, St. Louis. Both glyphosate and S3P were free of contaminants as judged by ^{1}H NMR spectroscopy. Protein size markers were from Amersham Pharmacia. Trypsin from bovine pancreas (12 081 units/mg) and glucose oxidase from *Aspergillus niger* (138 units/mg) were from Fluka, Switzerland. Protein concentration was determined using the Coomassie reagent from Pierce with BSA as standard according to the method reported in ref *21*.

Enzymes. UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) from *Enterobacter cloacae* was expressed in *E. coli* and purified as reported earlier (*8*).

The covalently bound intermediate of MurA, in which phosphoenolpyruvate is covalently attached to the side chain of cysteine 115, was synthesized and purified according to Wanke et al. (*8*). Protein purity and mass (observed mass, 44 778; calculated mass, 44 776) were checked by means of electrospray ionization-mass spectrometry as reported earlier (*8*).

For overexpression and purification of EPSPS, the open reading frame coding for EPSPS from *E. coli* was cloned as a PCR product into the expression vector pTRC 99A (Amersham Pharmacia) using the *Nco*I and *Bam*HI cloning sites. The protein was expressed in *E. coli* JM 105 (Amersham Pharmacia) according to the manufacturer's recommendations, and purified according to the protocol developed for MurA with the following alterations: Proteins precipitating between 35 and 70% $(NH_4)_2SO_4$ saturation were collected by centrifugation (30 min, 12000*g*, 4 °C) and resuspended in 50 mM Tris-HCl, 2 mM DTT, and 1 M $(NH_4)_2SO_4$. The solution was loaded onto a phenylsepharose column, equilibrated with the same buffer. Phenylsepharose and Q-sepharose chromatography were performed as described in (*8*) with 50 mM Tris-HCl, 2 mM DTT, 1 M $(NH_4)_2SO_4$ as the high salt buffer for phenylsepharose chromatography. Active fractions from the Q-sepharose column were pooled and aliquots of 50 mg of EPSPS were loaded onto a MonoQ column (HR16/10, Amersham Pharmacia) equilibrated with 50 mM Tris-HCl and 2 mM DTT, pH 7, at a flow rate of 7.5 mL/min. A linear gradient of 0 to 0.4 M KCl in 5 bed volumes was applied to develop the column. Electrospray ionization-mass spectrometry was used to determine purity and appropriate mass (observed mass, 46 092; calculated mass, 46 095; according to EPSPS database entry P07638) of the protein.

Tryptic Digests. With the exception of MurA C115S, which was incubated with ligands for 1 h before trypsin was added, enzyme was preincubated for 10 min with ligands before proteolysis was started.

Tryptic Digestion of UDP-N-acetylglucosamine Enolpyruvyltransferase (MurA) and 5-Enolpyruvylshikimate 3-Phosphate Synthase (EPSPS) and Analysis by SDS−PAGE. MurA (50 μM) was incubated with either a low or a high trypsin concentration (0.05 and 0.25 mg/mL, respectively) either in the absence or presence of the following reagents (1 mM final concentration): fosfomycin, UDPNAG, UDP-glucose, and PEP, single or in combination for 120 min at 25 °C. Incubation was carried out in 50 mM Tris-HCl and 1 mM DTT, pH 7.4. Twenty-microliter aliquots of the incubation mix were transferred to an Eppendorf tube and mixed with 20 μL of 2 × SDS-Laemmli sampling buffer, heated for 5 min at 95 °C, centrifuged 5 min at maximum speed in a benchtop centrifuge and an aliquot of 0.4 μL was loaded on a 12.5% SDS−polyacrylamide gel.

EPSPS (50 μM) was incubated with either a low or a high trypsin concentration (0.05 and 0.25 mg/mL, respectively), either in the absence or presence of the following reagents: glyphosate, S3P, shikimate, and PEP, single or in combination, for 180 min at 25 °C in 50 mM Tris-HCl and 1 mM DTT, pH 6.9. To quench residual trypsin activity after SDS denaturation, 20-μL aliquots of the incubation mix were transferred to an Eppendorf tube, mixed with 20 μL of 10% (v/v) trifluoroacetic acid and the precipitate pelleted at maximum speed in a benchtop centrifuge for 30 min. The supernatant was discarded, and the pellets were resuspended in 20 μL of Laemmli sample buffer. The basic pH of the sample buffer was restored by addition of 1 μL of 6 M NaOH. The following procedure was as described for MurA, except that 20% SDS−polyacrylamide gels were used in order to detect small proteolytic fragments.

SDS−PAGE was carried out as described (*22*), using PHAST-gels and the PHAST system from Amersham Pharmacia in the appropriate modes. The protein bands were visualized by Coomassie Brillant Blue R250 staining.

Measurement of Residual Activity Following Tryptic Digestion. Proteolysis of MurA (100 μM) was carried out in 50 mM Tris-HCl and 1 mM DTT, pH 7.4, with a final trypsin concentration of 0.5 mg/mL. At the indicated time points (see figure legends), 10 μL aliquots were diluted 1/100 in the test buffer to measure residual activity using an enzyme-coupled assay. In this assay, the product enolpyruvyl-UDPNAG is enzymatically reduced by UDP-*N*-acetylenol-pyruvylglucosamine reductase (MurB). This reaction proceeds at the expense of reduced NADP and can be followed at 340 nm in a spectrophotometer as described (*23*, *24*).

Proteolysis of EPSPS was carried out with 0.1 μM EPSPS in 50 mM Tris-HCl and 1 mM DTT, pH 6.9, with 0.5 mg/mL trypsin. At given time points, aliquots were taken and diluted 10-fold with test buffer containing 1 mM S3P and 1 mM PEP. Residual activity of EPSPS was measured based on the rate of release of inorganic phosphate according to the method reported by Lanzetta et al. (*25*).

The reference value (100% activity) was for both enzymes the activity determined for a sample taken immediately after addition of trypsin. Data were fitted using SigmaPlot (Jandel Scientific) v4.14, according to the following equation:

$$V_{res}(t) = a \exp(-bt) + c \exp(-dt)$$

with V_{res} (residual activity) as function of time (t).

MALDI-TOF-MS Analysis of MurA and EPSPS Tryptic Fragments. MurA at a concentration of 5 mg/mL (ca. 100 μM) with the additions as denoted, was incubated with 0.5 mg/mL trypsin at 25 °C in 50 mM Tris-HCl and 1 mM DTT. Ten microliters of the incubation mixture was diluted with 40 μL of 10% trifluoroacetic acid to quench the proteolytic activity of trypsin. One microliter of this sample was deposited on a MALDI sample plate and mixed with 1 μL of a saturated dihydroxybenzoate solution in 0.1% trifluoroacetic acid/acetone (2:1, v/v). EPSPS was treated identically except that the incubation buffer was at pH 6.9.

MALDI-TOF-MS spectra of the peptide fragments were recorded with a Voyager Elite mass spectrometer using the

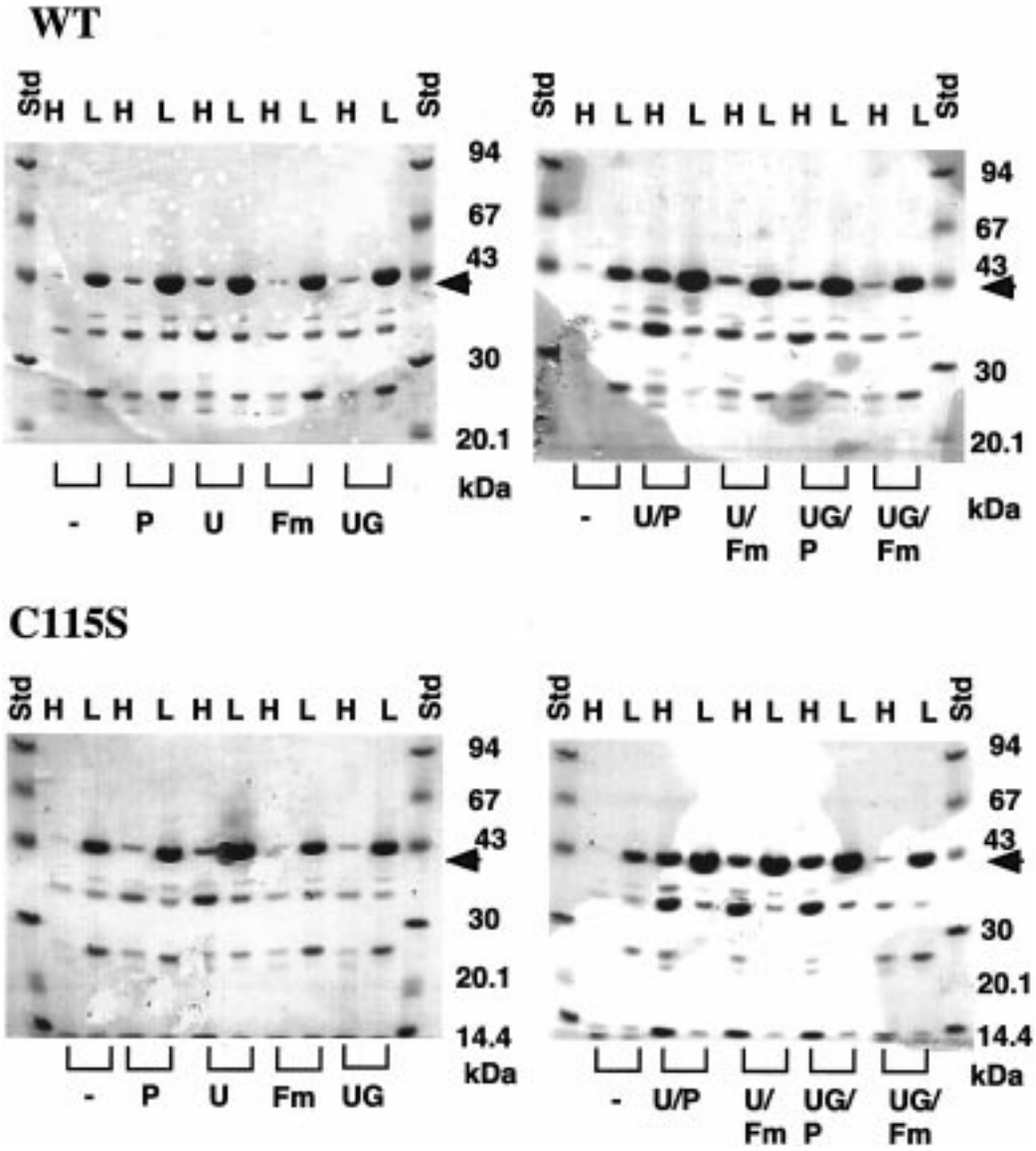


FIGURE 1: SDS−gel electrophoresis of tryptic digests of wild-type MurA (top panels) and the C115S mutant protein (bottom panels). MurA (50 μM) was incubated for 2 h at 25 °C using two different trypsin concentrations: 0.25 mg/mL (H) and 0.05 mg/mL (L), as indicated on top of each gel. Additions (1 mM) are indicated below each gel: P, phosphoenolpyruvate; U, UDPNAG; UG, UDP-glucose; and Fm, fosfomycin. The arrowheads indicate the apparent molecular mass of undigested MurA.

reflectron mode for increased mass accuracy. Analysis of the peptide masses was performed using the program PAWS v. 8.1.1 (freeware edition for MacOs 7.5, copyright Proteo-Metrics, 1997).

Immune Precipitation with Monoclonal Antibodies against EPSPS. The monoclonal antibody MCA-1 was generated using standard protocols as described in detail in ref *20* (originally designated as "MAk 41-6-A1"). Purified native EPSPS from *E. coli* strain NS 260 (*26*) was used for immunization. Briefly, MCA-1 was obtained from the supernatant of the culture medium of mouse hybridoma cells. Determination of the immunoglobulin class was performed using MonoAB-ID EIA kit (Zymed, Wiesbaden, Germany). According to this analysis, MCA-1 belongs to the immunoglobulin G class and, hence, exhibits two antigen-binding sites per antibody molecule. The concentration of the antibody was determined by titration with the antigen (EPSPS) followed by precipitation of the MCA-1/EPSPS-complex and measuring the residual enzyme activity in the supernatant. Using this procedure, a MCA-1 concentration of 19 ng/mL (or 256 nM with respect to antigen binding sites) was obtained.

EPSPS (200 ng) or EPSPS ligand complexes (1 h preincubation) were immunoprecipitated with MCA-1 for 3.5 h at room temperature by incubation with protein A crude cell suspension for 2 h (4% v/v, Sigma) followed by incubation with rabbit-anti-mouse-immunoglobulin (8% v/v, Sigma) for 1.5 h. Incubation was performed in a total volume of 150 μL of 50 mM Tris/HCl, pH 8, containing 150 mM NaCl, 0.025 vol % Tween 20, 0.5 g of BSA/mL. Immunoprecipitated proteins were collected by centrifugation at 12 000 rpm in a microfuge. Residual EPSPS activity in the supernatant was used to calculate the fraction of immunoprecipitated enzyme.

To determine the dissociation constant of MCA-1 for EPSPS and the EPSPS ligand complexes (1 h preincubation), increasing amounts of the respective antigen (50−1000 ng) were incubated with a constant amount of MCA-1 followed by immunoprecipitation. Residual enzyme activity in the supernatant, compared to controls without MCA-1, was used

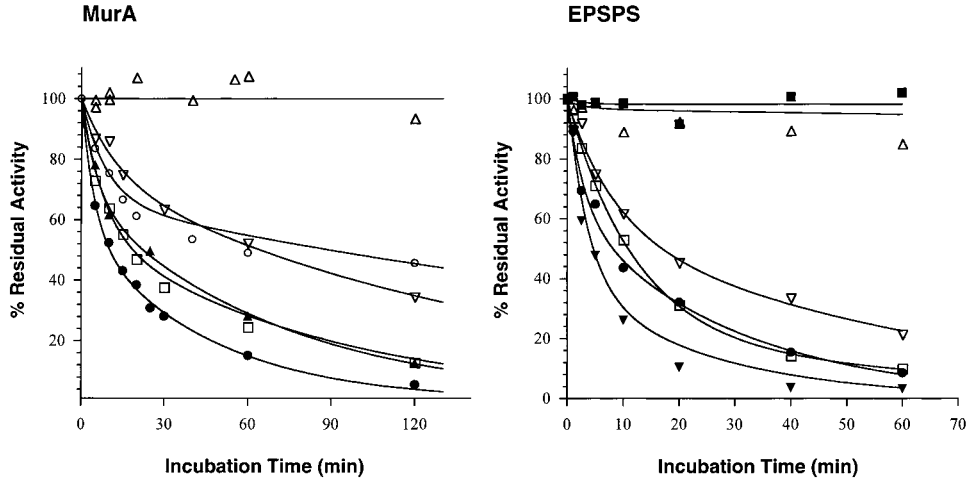


FIGURE 2: (Left panel) Residual MurA activity during tryptic digestion (trypsin concentration, 0.5 mg/mL) as a function of substrate presence and concentration. The symbols represent measurements during the course of the experiment under the following conditions: (△) 1 mM PEP and 1 mM UDPNAG; (○) covalent intermediate; (▽) 1 mM UDPNAG; (▲) 0.1 mM PEP and UDPNAG; (□) 1 mM PEP; (●) no additions. (Right panel) Residual EPSPS activity during tryptic digestion. EPSPS (0.1 μM) was digested with trypsin (0.25 mg/mL) at 25 °C in the presence of the following ligands: (■) S3P (1 mM) and glyphosate (10 μM); (△) S3P (1 mM) and PEP (1 mM); (▽) S3P (1 mM); (□) PEP (1 mM); (●) no additions, and (▼) glyphosate (10 μM). The data were fitted using the equation $f(x) = a \exp(-bx) + c \exp(-dx)$, with $a + c =$ the initial activity and b, d the time constants of enzyme inactivation.

to determine the concentration of free EPSPS. The maximal antibody-bound enzyme concentration represents the concentration of antibody-binding sites in this assay. This value was used to calculate the free antibody concentration shown in Figure 9.

RESULTS

Protection of UDP-N-acetylglucosamine Enolpyruvyl Transferase (MurA) against Tryptic Digestion. Tryptic digestion of MurA was first analyzed by denaturing polyacrylamide gel electrophoresis (Figure 1). Depending on the concentration of trypsin, wild-type MurA as well as C115S mutant protein was digested completely (high trypsin concentration = 0.25 mg/mL) or partially (low trypsin concentration = 0.05 mg/mL) within 2 h as shown in Figure 1 (left two lanes in each panel). Single addition of either a substrate, a substrate analogue, or the inhibitor fosfomycin had only a moderate effect on the digestibility of the protein (Figure 1, top left panel). This was also found with the C115S mutant protein (Figure 1, bottom left panel). On the other hand, combinations of these ligands resulted in reduced digestibility of the protein (Figure 1, top right, wild-type; bottom right, C115S mutant protein). The most effective protection was observed with the combination of the natural substrates PEP and UDPNAG and by UDPNAG and fosfomycin. A slightly smaller effect was seen with the combination of UDP-glucose and PEP, while the simultaneous presence of UDP-glucose and fosfomycin seems to exert only a small effect (Figure 1, right two lanes in the top and bottom right panels).

To obtain more quantitative data on the protective effect of the substrates, PEP and UDPNAG, residual enzyme activity was determined as a function of substrate concentration and incubation time with trypsin, in the high concentration according to Figure 1. In comparison with free MurA, UDPNAG alone (at 1 mM concentration) protects MurA more effectively than PEP alone (Figure 2, left panel). In the presence of both substrates UDPNAG and PEP, the degree of protection depends strongly on the concentrations of substrates used. In general, the loss of activity was found to proceed biphasically and could be fitted to a double-exponential rate equation.

Analysis of a tryptic digest of MurA or EPSPS by means of nondenaturing PAGE using an activity staining for phosphate release indicated that only the intact protein with an apparent molecular mass of 44 kDa, the approximate molecular mass of MurA and EPSPS, respectively, had activity (data not shown). The resolution of this method, however, does not rule out that smaller peptide fragments, i.e., in the mass range of 1−5 kDa, can be cleaved without a loss of enzymatic activity.

While the reactions of both MurA and EPSPS proceed through a tightly but noncovalently bound tetrahedral intermediate, MurA is capable of forming an additional covalent adduct with PEP (the *O*-phospho-thioketal of pyruvic acid, bound to C115) in the presence of UDPNAG (*8*, *23*, *27*). The role of this covalent intermediate is discussed extensively (*10*, *23*). Interestingly enough, the covalent phosphoenolpyruvate-MurA adduct provides protection against trypsin digestion (Figure 2, open circles). The degree of this protection is similar to that found in the presence of 1 mM UDPNAG.

MALDI-TOF-MS Analysis of Peptide Fragments Obtained from Tryptic Digestion of UDP-N-acetylglucosamine Enolpyruvyltransferase (MurA). The experiments described in the previous section provide qualitative evidence that the conformational changes taking place in MurA upon binding of substrates, substrate analogues or the inhibitor fosfomycin are accompanied by a reduced susceptibility toward proteolysis. To gain site-specific information on the susceptibility of certain regions of MurA toward tryptic digestion, the appearance of peptide fragments was analyzed during the course of trypsin digestion. In the absence of any ligands, MurA is readily digested, yielding 22 peptide fragments covering 81.6% of the total MurA sequence (see Figure 3, panel A, and Table 1). During the course of the digestion, the first observable peptides have monoisotopic molecular masses of 1142, 1254, 1933, 2025, and 2561 kDa and were assigned to the fragments designated Q, E, F, T, R, and C

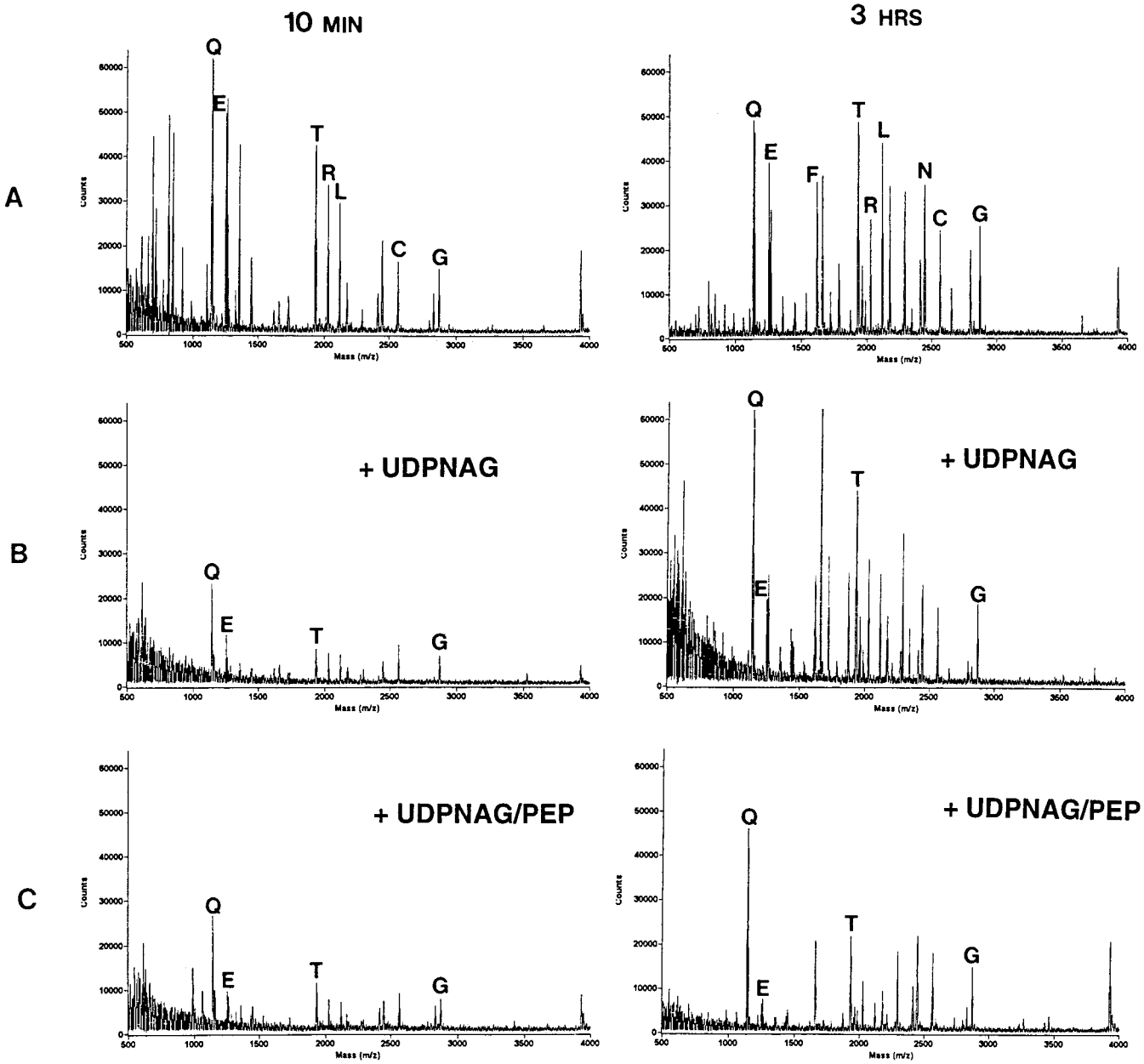


FIGURE 3: Appearance of peptide fragments during the course of tryptic digestion of MurA as analyzed by MALDI-TOF mass spectrometry. The mass spectra after 10 min (left panels) and 3 h (right panels) are shown. MurA (100 μM) was incubated with 0.5 mg/mL trypsin without additions (A), in the presence of 1 mM UDPNAG (B) and 1 mM UDPNAG + 1 mM PEP (C) at 25 °C.

(see Table 1). Inspection of the 3D-structure of MurA [cf. Figure 10 (*3*, *10*)] reveals that these peptide fragments are generated by tryptic cleavage in the loops of folding unit Ia (T), Ic (Q and R), IIb (C), and IIc (E and F), respectively. In the open form of the enzyme, the loop in the folding unit IIc is accessible, while in the closed form, this loop (catalytic loop) has moved toward the active center of the enzyme and is part of a more tightly packed structure (cf. Figure 10). On the other hand, the other areas mentioned above are not (directly) involved in catalysis and do not undergo a similar rearrangement.

When MurA was digested with trypsin in the presence of UDPNAG (Figure 3, panel B) or UDPNAG and PEP (Figure 3, panel C), the appearance of most peptides was considerably retarded as compared to free, unliganded MurA (Figure 4, panels A−C). It is noteworthy that in the presence of UDPNAG, especially the appearance of fragments E and F is suppressed. This effect was more pronounced in the presence of both substrates. The only exceptions observed from this general tendency were the peptide fragments Q and to a lesser extent peptide fragment T. These peptides originate from tryptic cleavage in the loop region of folding units Ia and Ic, respectively. Unlike the catalytic loop of folding unit IIc, these regions are not directly involved in the catalytic processes (*10*), suggesting that binding of substrates provides a higher degree of shielding for the catalytic loop. Further insight concerning the protection of the loop regions was obtained with the catalytically inactive C115S mutant protein. As mentioned above, in support of the results shown in Figure 1, the rate of appearance of most peptide fragments was reduced in the presence of UDPNAG and PEP (Figure 4, panel C). In the case of the C115S mutant protein, however, not only the peptide fragment Q but especially peptide fragment G ($MH^+ = 2851$) was found to

Table 1: MALDI-TOF-MS Analysis of Fragments Obtained from Trypsin Digestion of MurA

fragments (aa positions)	label	domain	secondary structure	molecular mass (calculated mi MH^+)	molecular mass (observed mi MH^+)	% of total sequence
6−11	A	Ia	β/loop	657.4	658	1.4
12−22	B	Ib	β/loop	1102.6	1103	2.6
23−46	C	IIb	α/loop/β/loop	2560.4	2561	5.7
67−88	D	IIb/IIc	loop/β/loop/β/loop	2410.1	2410	5.2
92−103	E (▲)	IIc	α	1253.7	1254	5.2
104−120	F (□)	IIc	loop/α/loop	1618.9/1602.8[a]	1618/1602[a]	4.0
104−131	G (△)	IIc	loop/α/loop/β	2866.5/2850.4[a]	2868/2850[a]	6.7
132−144	H	IIc	loop/β/loop/β	1448.8	1449	5.5
145−152	I	IIc	β/loop	844.5	844 (?)	1.9
161−187	J	IIa	loop/α/loop/β/loop	2647.4	2647	4.0
188−204	K	IIa	α/loop	1786.9	1787	4.0
228−248	L (■)	IIb	loop/α/loop	2115.2	2115	5.0
253−265	M	Ib	loop/α	1355.7	1356	3.1
266−287	N	Ib	α/loop/β/loop/β/loop	2443.2	2444	5.2
266−290	O	Ib	α/loop/β/loop/β/loop	2824.4	2825	6.0
296−331	P	Ic	loop/α/loop/β/loop	3930.0	3932	8.6
332−340	Q (○)	Ic	loop/α	1141.6	1142	2.1
341−359	R (▽)	Ic	loop/β/loop/β/loop	2025.0	2025	4.5
360−371	S (●)	Ia	loop/β/loop	1261.7	1262	2.9
372−391	T (▼)	Ia	α/loop/β	1933.0	1934	4.8
392−401	U	Ia	loop	1321.7	1322	2.4
408−415	V	Ia	loop/β	843.4	844 (?)	1.9
						Σ = 81.6

[a] masses for the C115S mutant protein; aa position, amino acid sequence numbering starting with the N-terminus; (?) indicates the ambiguity in assignment of fragments I and V; mi, monoisotopic masses; symbols in parentheses indicate those used in Figure 4.

appear faster during the time course of the digestion (Figure 4, compare panels C and D). Interestingly enough, in the case of wild-type MurA, fragment G appears only transiently and is rapidly digested to the smaller fragment F by cleavage of the peptide at arginine 120 (cf. Figure 10 for structural details).

Tryptic digestion of MurA in the presence of the antibiotic fosfomycin and UDPNAG resulted in an extremely slow proteolysis (Figure 5). Even after 20 h of incubation at 25 °C, only two peptide fragments could be identified as fully cleaved tryptic fragments, i.e., peptides with monoisotopic molecular masses of 1142 (Q) and 2026 (R), respectively. Both peptide fragments result from tryptic cleavage in the loop region of folding units Ia and Ic (see Table 1). The protective effect of fosfomycin and UDPNAG exceeds that observed with PEP and UDPNAG, indicating that fosfomycin locks MurA in both a catalytically inactive and tightly packed structure. In this structure, the most accessible cleavage sites are in the folding unit Ic whereas access to the cleavage sites in the catalytic loop region appears to be severely restricted. This result is qualitatively supported by the electrophoretic analysis as shown in Figure 1, although, contrary to the results in Figure 1, mass spectrometric analysis suggests that fosfomycin/UDPNAG exhibits a more pronounced protective effect than PEP/UDPNAG. This was interpreted as the result of continuing proteolysis after sample denaturation in SDS−PAGE loading buffer; for this reason, the tryptic digests of EPSPS were quenched with trifluoroacetic acid prior to dilution in loading buffer.

Protection of 5-Enolpyruvylshikimate 3-Phosphate Synthase (EPSPS) against Tryptic Digestion. Tryptic digestion of EPSPS under various conditions was first studied by SDS−PAGE (Figure 6). Unliganded EPSPS appears to be less susceptible to tryptic digestion (Figure 6, left two lanes in both panels) than MurA (compare with left two lanes in Figure 1, upper panels). Although the presence of either PEP (P), S3P, or glyphosate (Glp) appeared to moderately slow tryptic digestion, especially with S3P, major effects were only found in the presence of combinations of some of these ligands. The most pronounced shielding was found in the presence of the two substrates, i.e., S3P and PEP or S3P and glyphosate (see Figure 6, right panel). The SDS−PAGE gel in Figure 6 also shows that tryptic digestion of EPSPS leads to two dominant fragments with approximate masses of 36 000 and 8000 Da. These are found under all conditions, except for the experiment performed in the presence of S3P and glyphosate, emphasizing the strong cooperative effect of these two ligands.

Like with MurA, the protection against tryptic digestion of EPSPS was monitored by measuring residual enzyme activities as a function of incubation time with trypsin. In this experiment, a higher degree of resistance toward trypsin became obvious: while in the corresponding experiment with MurA, a concentration of 100 μM of MurA and 0.5 mg/mL trypsin was used, the ratio of trypsin to EPSPS had to be 1000-fold higher to achieve the same degree of enzyme inactivation (ca. 10% residual activity). As shown in Figure 2 (right panel), the presence of either both S3P and glyphosate or both S3P and PEP results in almost complete conservation of enzyme activity during the course of the experiment. The presence of S3P led to only a modest protection of enzyme activity. On the other hand, addition of PEP had no effect, and the presence of glyphosate even seemed to enhance the rate of proteolytic inactivation. Again, as was observed for MurA, PEP had a smaller effect than the enolpyruvyl-accepting substrate (compare left and right panels of Figure 2). In all cases, the effects of the addition of one of the substrates alone was less pronounced.

MALDI-TOF-MS Analysis of Peptide Fragments Obtained from Tryptic Digestion of 5-Enolpyruvylshikimate 3-Phosphate Synthase (EPSPS). As shown above, compared to MurA, EPSPS is much more stable toward tryptic digestion

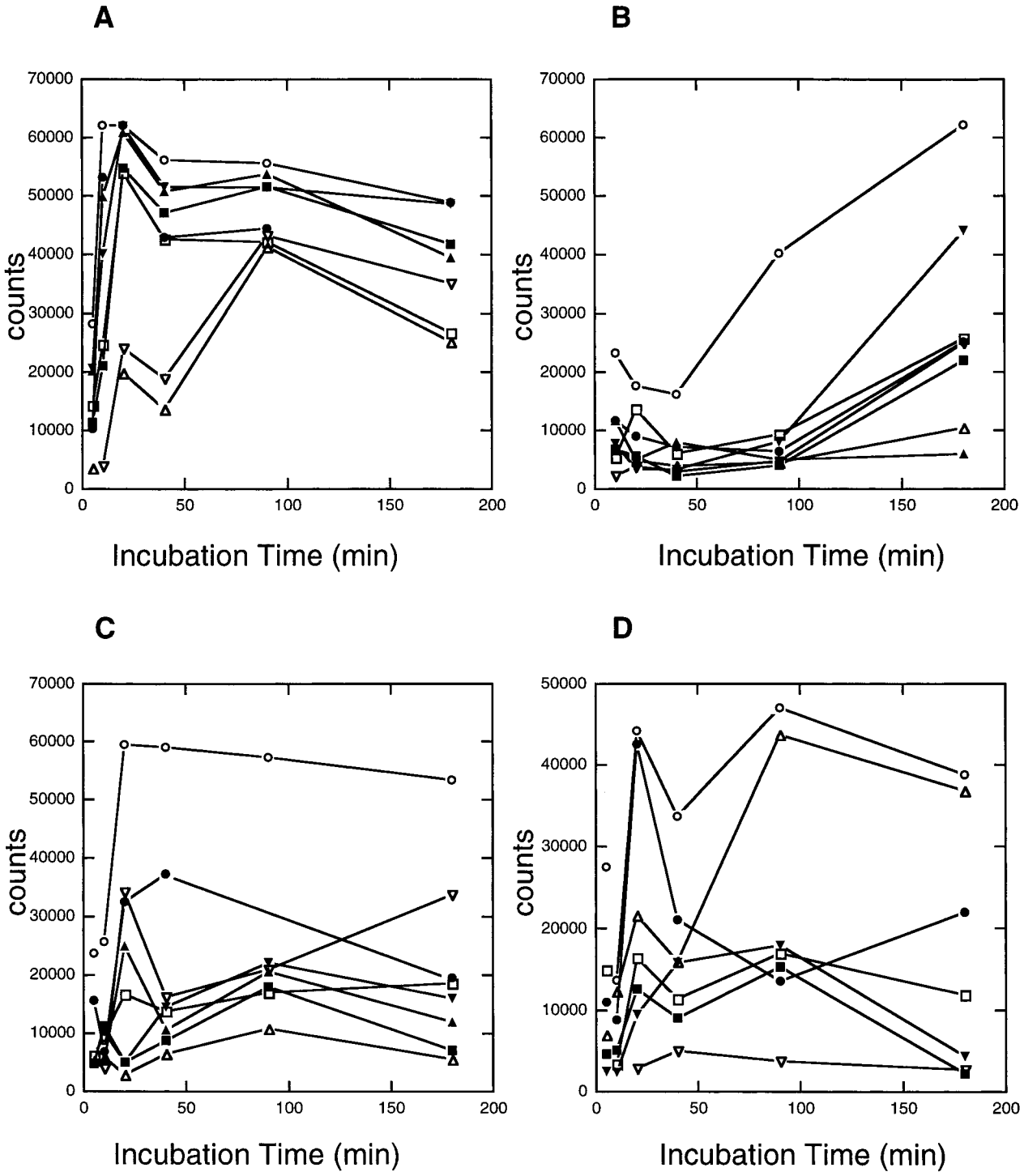


FIGURE 4: Appearance of peptide fragments during the course of tryptic digestion as analyzed by MALDI-TOF mass spectrometry. All experiments were performed at concentrations of MurA of 100 μM and trypsin of 0.5 mg/mL, respectively. (A) unliganded MurA; (B) MurA in the presence of UDPNAG (1 mM); (C) MurA in the presence of UDPNAG and PEP (1 mM each); (D) C115S mutant protein in the presence of UDPNAG and PEP (1 mM each). The symbols for the peptide fragments are as follows: (▲) fragment E ($MH^+ = 1254$); (△) fragment G ($MH^+ = 2868$ and 2850 for wild-type MurA and the C115S mutant protein, respectively); (□) fragment F ($MH^+ = 1618$ and 1602 for wild-type MurA and the C115S mutant protein, respectively); (■) fragment L ($MH^+ = 2115$); (○) fragment Q ($MH^+ = 1142$); (▽) fragment R ($MH^+ = 2025$); (●) fragment S ($MH^+ = 1262$), and (▼) fragment T ($MH^+ = 1934$).

than MurA. The MALDI-TOF mass spectrum (Figure 7) is dominated by three peptide fragments with observed molecular masses of 1377, 1475, and 1637 (MH^+), labeled 1, 2, and 3, respectively. For each of the first two peptides, only one tryptic fragment matching the observed masses could be identified using the program PAWS (±2 ppm). According to this analysis, the two peptides comprise the amino acids 123−134 and 355−367, respectively, which have calculated MH^+ peaks of 1376 and 1474, respectively. The third peak at $MH^+ = 1637$, however, could not be unambiguously assigned by this method, since three tryptic fragments were found to be within ±2 ppm of the observed mass. The identity of this peptide was established using MS/MS electrospray generating a partial sequence which fits to a peptide fragment having the mass of 1635.8 Da and comprises amino acids 140−152. Fragment 1 includes the

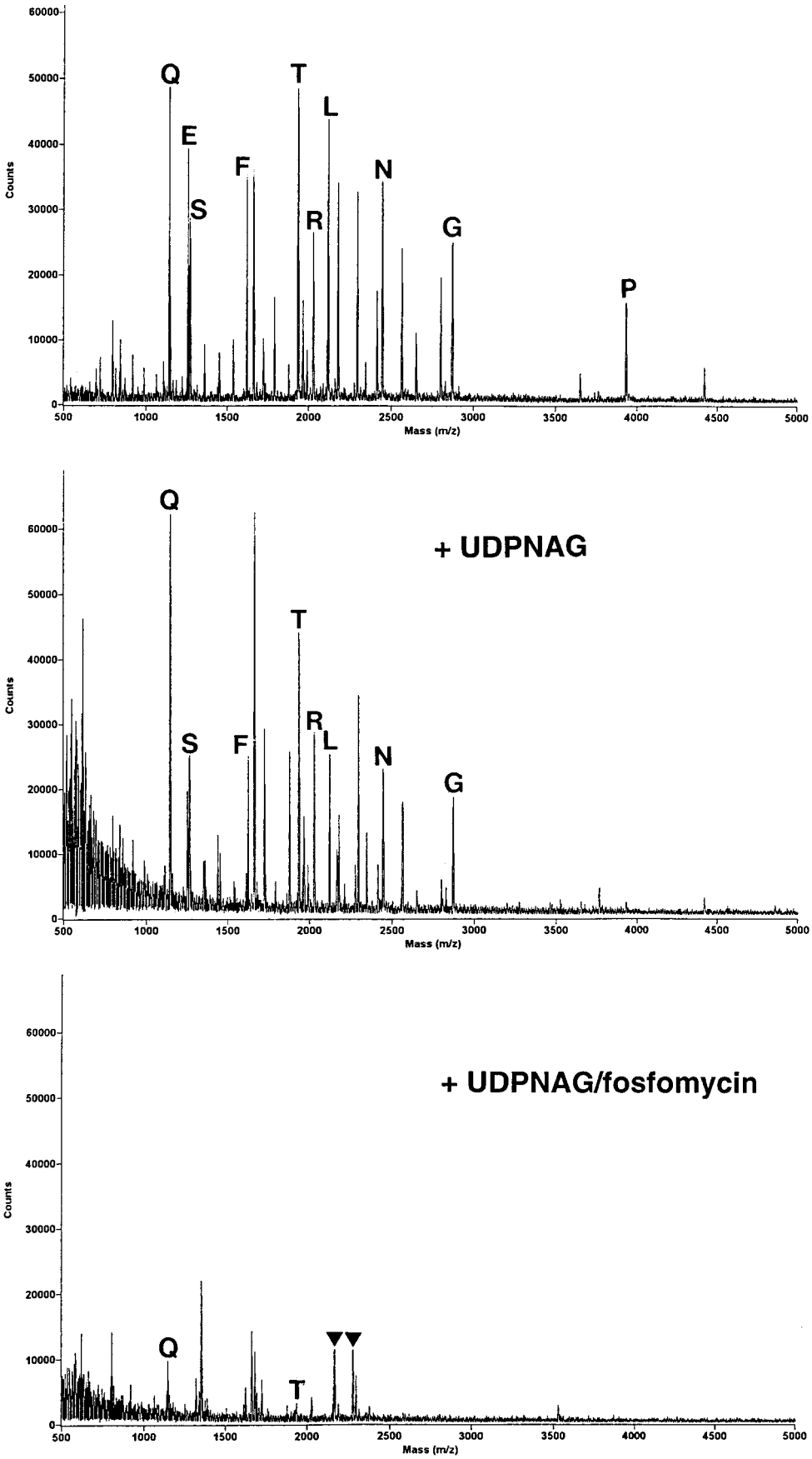


FIGURE 5: Protection of MurA against tryptic digestion in the presence of UDPNAG and fosfomycin. The mass spectra show the detectable peptide fragments released after 3 h of digestion in the presence of 0.5 mg/mL trypsin (top panel), in the presence of 0.5 mg/mL trypsin and UDPNAG (1 mM) (central panel) and in the presence of 0.5 mg/mL trypsin, UDPNAG (1 mM), and fosfomycin (1 mM) (bottom panel). Autocleavage fragments of trypsin are labeled with triangles.

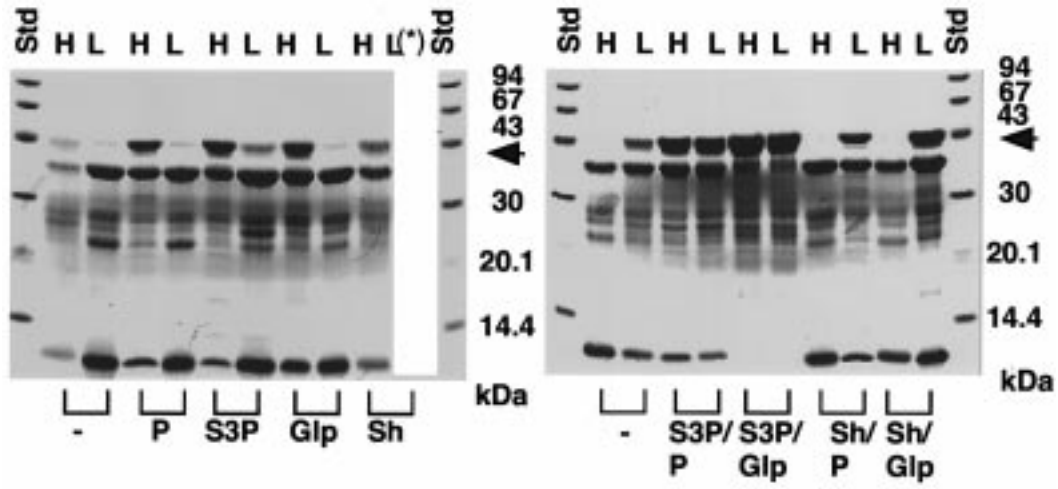


FIGURE 6: SDS−PAGE analysis of tryptic digests of EPSPS. EPSPS (100 μM) was incubated for 3 h at 25 °C using two different trypsin concentrations: 0.25 mg/mL (H) and 0.05 mg/mL (L), as indicated on top of each gel. Additions (1 mM) are indicated below each gel: P, phosphoenolpyruvate; S3P, shikimate-3-phosphate; Glp, glyphosate and Sh, shikimate. The arrowheads indicate the apparent molecular mass of undigested EPSPS. (*) No data available.

short α-helical stretch of EPSPS that corresponds to the catalytic loop in MurA. Fragment 2 is located in a loop region in domain Ic and corresponds to the noncatalytic fragment R in MurA. Like fragment 1, fragment 3 is located in domain IIc. In MurA, the corresponding structural element comprises a β-sheet region near the active-site loop.

In the presence of the substrate shikimate 3-phosphate (S3P), the appearance of these peptides during the course of tryptic digestion is reduced, similar to the findings with MurA in the presence of UDPNAG. In the presence of the herbicide glyphosate and S3P, no tryptic peptide fragments could be observed, indicating that the trypsin cleavage sites had become inaccessible. This result is similar to the tryptic digestion of MurA in the presence of fosfomycin and UDPNAG. Hence, glyphosate and fosfomycin in conjunction with the respective substrate exhibit comparable effects on the susceptibility of these proteins toward tryptic digestion. In other words, both ligands induce a tightly packed protein structure rendering tryptic cleavage sites basically inaccessible.

Immune Precipitation of 5-Enolpyruvylshikimate 3-Phosphate with a Monoclonal Antibody. The monoclonal antibody (MCA-1), generated against native EPSPS, led to complete precipitation of the protein resulting in reduced enzyme activity in the supernatant (Figure 8). Addition of PEP had only a minor effect on the precipitation, while addition of EPSP or S3P and glyphosate increased the residual enzyme activity strongly (see Figure 8). From the data shown in Figure 8, the concentration of bound and free EPSPS was calculated and the dissociation constants (K_D) under the various experimental conditions were determined in a double reciprocal plot (Figure 8, inset). The K_D of free EPSPS and MCA-1 is 9 nM whereas addition of EPSP increases the K_D to 54 nM with a further increase to 180 nM in the presence of S3P and glyphosate. To characterize the nature of the reduced antibody affinity in the presence of S3P and glyphosate, residual enzyme activity was determined as a function of glyphosate concentration, in the presence of a saturating concentration of S3P (1 mM). A double reciprocal plot of the concentration of antibody-bound EPSPS versus the antibody concentration yields a family of lines as shown in Figure 9. A replot of the slopes of the lines versus the glyphosate concentration is shown in the inset of Figure 9. The hyperbolic function indicates that binding of glyphosate and the antibody to EPSPS is only partially competitive, i.e., binding is not mutually exclusive. In other words, MCA-1 binds to both free EPSPS and the ternary complex of EPSPS·S3P·glyphosate, albeit with a much higher K_D in the latter case. Qualitatively, the same result was obtained in the presence of EPSP. The demonstration that the binding affinity of MCA-1 is affected by the presence of EPSP or S3P/glyphosate can be interpreted along the same lines as the proteolysis experiments described in the previous sections: binding of EPSP or S3P/glyphosate induces a conformational change which in turn diminishes the interactions of EPSPS with the antibody giving rise to an increased K_D for the complex.

DISCUSSION

The study of limited tryptic digestion of MurA and EPSPS has demonstrated that, with respect to their sensitivity to proteolysis, these two structurally and mechanistically related enzymes also respond similarly to binding of substrates and inhibitors. In the case of MurA, structural studies have revealed the existence of (at least) two conformations: in the free unliganded state, MurA exists in an open form with an extended cavity in the central part of the macromolecule (*3*) (left structure in Figure 10). Binding of UDPNAG and, for example, the antibiotic fosfomycin results in a closed conformation (central structure in Figure 10) (*10*). This conformational change involves rearrangment of the hydrogen bond interactions in the upper domain of the protein as well as a movement of a loop region (catalytic loop) in this domain toward the cavity forming the active center (compare left and central structures in Figure 10). Our study has shown that conditions leading to the formation of the closed conformation of MurA also result in an increased stability toward tryptic digestion (Figures 1−5). Although the protection induced by ligand binding appears to be largely unspecific, site-specific effects in the proteolytic susceptibility were identified by means of mass spectrometric analysis of the peptide fragments. The peptide fragments derived from

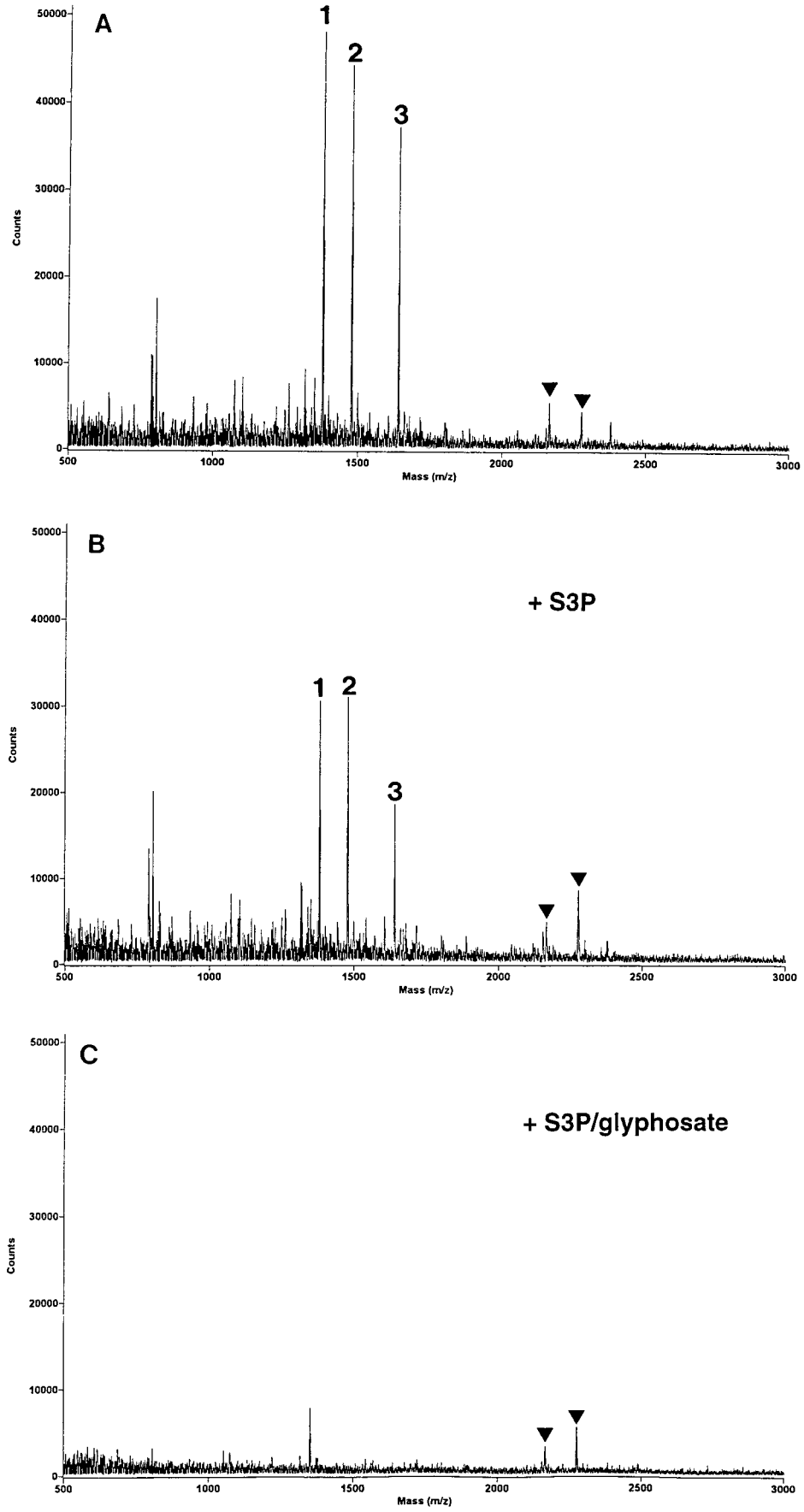


FIGURE 7: Tryptic digestion of *E. coli* EPSPS in the absence of ligands (panel A) and in the presence of 1 mM S3P (panel B) and 1 mM each S3P/glyphosate (panel C), respectively. Shown are the MALDI mass spectra after 3 h of incubation with 0.5 mg/mL trypsin (final concentration) at 25 °C. The peaks labeled with 1, 2, and 3 have monoisotopic masses (MH^+) of 1376.8 (amino acids 123−134), 1474.8 (amino acids 355−367), and 1637.0 (amino acids 140−152), respectively. The peaks labeled with triangles are trypsin autocleavage fragments.

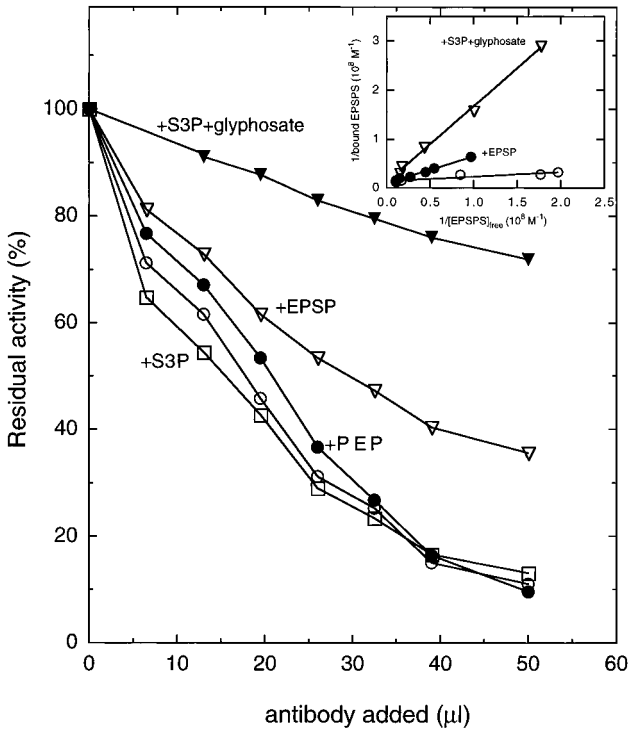


FIGURE 8: Immune precipitation of EPSPS employing monoclonal antibody MCA-1. Residual EPSPS activity was determined after precipitation with the monoclonal antibody MCA-1 as described in the Materials and Methods. The data shown are from the following experiments: (○) no addition; (□) S3P (1 mM); (●) PEP (10 mM); (▽) EPSP (0.2 mM); and (▼) S3P (1 mM) and glyphosate (20 μM). The inset shows a titration of MCA-1 with EPSPS as a double reciprocal plot of antibody-bound versus free EPSPS as determined by immunoprecipitation with MCA-1 (950 ng). The concentration of free and antibody-bound EPSPS was calculated as described in the Materials and Methods. From these data, dissociation constants for EPSPS-antibody complexes were calculated: in the absence of ligands (○) 9 nM, in the presence of EPSP 54 nM (●), and in the presence of S3P and glyphosate 180 nM (▽).

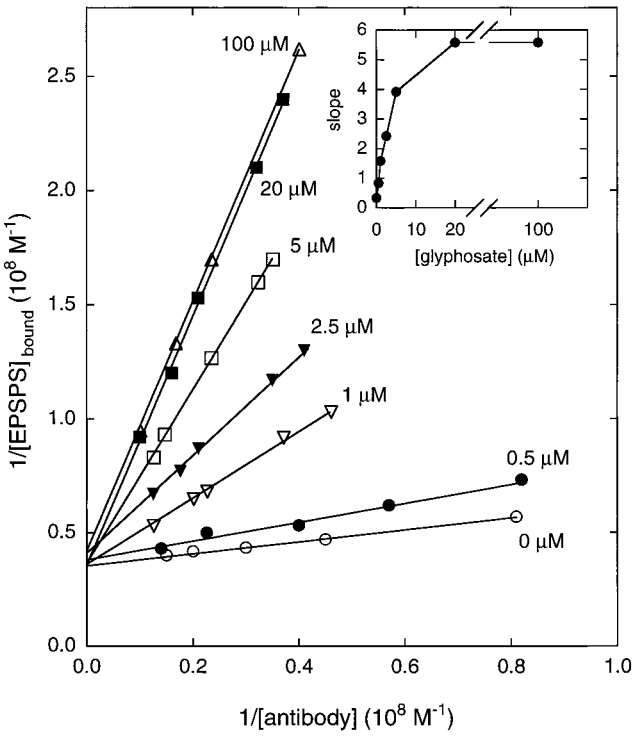


FIGURE 9: Immunoprecipitation of EPSPS with MCA-1 as a function of glyphosate concentration shown as a double reciprocal plot of antibody-bound EPSPS versus free antibody concentration. The concentration of S3P was 1 mM and the concentration of glyphosate was varied as indicated. The inset shows a replot of the slope of the obtained lines against the glyphosate concentration.

tryptic cleavage in the catalytic loop region are more protected than those derived from other loop regions. Quite surprisingly, in the catalytically inactive mutant protein C115S, this does not seem to be the case. It appears that the cysteine to serine exchange in this position not only annihilates the assumed general acid function (*8*, *23*) of the amino acid side chain but also compromises the ability of the loop to respond to substrate binding. In this context, it is noteworthy that noncovalently bound PEP, as well as PEP covalently bound as its *O*-phosphothioketal to the side chain of this cysteine, exerts a shielding effect against tryptic digestion (see Figures 1 and 2). This suggests that binding of PEP, in any of the two binding forms, gives rise to a "partially" closed conformation. It should be noted that partial protection against proteolysis in the presence of a single substrate species suggests the existence of partially closed conformations in the binary complexes. The presence of both substrates, i.e., formation of the ternary complex, then leads to the fully closed conformation and the pronounced synergistic effect on digestibility.

Comparative experiments with EPSPS have clearly shown the similarity of the two proteins with respect to the effects of substrate and inhibitor binding on the proteolytic susceptibility. These results can be taken as evidence for the existence of open and closed conformations of EPSPS, corresponding to the two conformations described for MurA. It should be pointed out that the catalytic loop in MurA is replaced by a short α-helix in EPSPS, and it remains to be seen how this structural element participates in the formation of the proposed closed conformation.

This conclusion derived from limited proteolysis experiments is supported by corroborating evidence obtained by means of an immunochemical method. The monoclonal antibody MCA-1 raised against EPSPS showed a 20-fold higher dissociation constant in the presence of both S3P and glyphosate. EPSP alone increased the dissociation constant only 6-fold, and S3P alone did not affect at all the affinity of MCA-1 to EPSPS. This indicates that binding of both S3P and glyphosate to EPSPS induces a different conformation of the protein. This conformational change reduces the accessibility of the epitope and hence diminishes the binding affinity. Quite surprisingly, S3P does not appear to even partially induce this conformational state as the dissociation constant remains unaffected. This is in contrast to the results of the limited proteolysis (see Figures 2, 6, and 7), which suggested a partial conformational change leading to a moderate protection against proteolysis. One possible explanation is that S3P alone induces a conformational change, leading to the observed effects in the limited proteolysis experiments, which is distinct from the one occurring upon binding of both S3P and glyphosate and does not yield a diminished antibody affinity. Interestingly enough, Stepanek has recently reported on the discovery of an EPSPS antibody which selectively recognized the EPSPS·S3P binary complex, suggesting that a conformational change does occur upon binding of S3P alone (*20*).

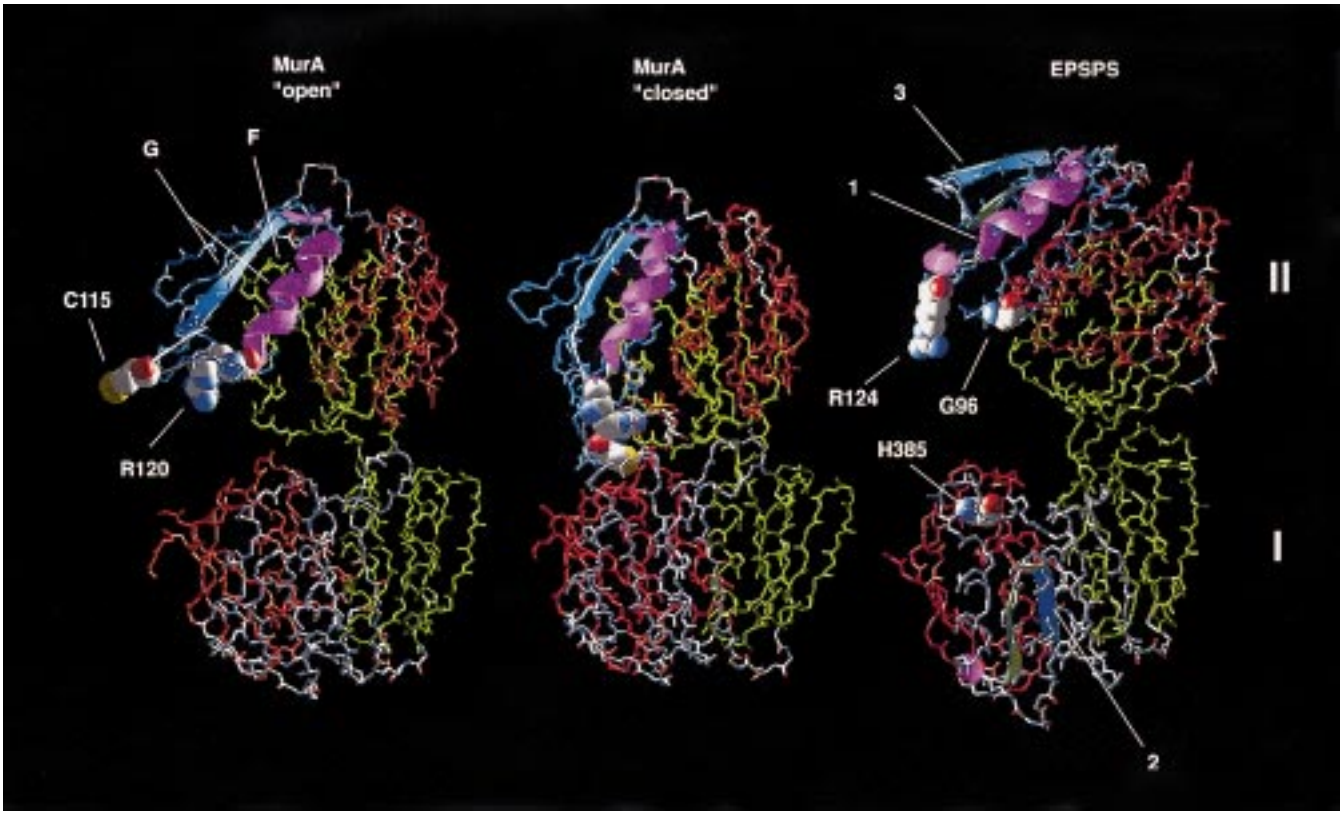


FIGURE 10: Three-dimensional illustration of the open and closed forms of MurA, left and central, and of EPSPS, right, showing the peptide backbone of the enzymes. The subdomains are marked in the color code used by Schönbrunn et al. (*3*): Domains Ia and IIa in red, Ib and IIb in green, and Ic and IIc in blue. In MurA, C115 and R120 are represented as spacefilling models; in EPSPS, G96, R124, and H385 are highlighted similarly. The peptide fragments F and G in MurA (Table 1), and fragments 1, 2, and 3 of EPSPS (Figure 7) are shown in ribbon representation. The picture was drawn using the SwissPDB viewer v 3.13 (*36*), utilizing PDB entries: 1NAW [MurA without ligands (*3*)], 1UAE [MurA complexed with UDPNAG and fosfomycin (*10*)], and 1EPS [(*2*), reconstructed in program O (*37*)].

Although MurA and EPSPS displayed many striking similarities in this study, it was observed that free MurA is much more sensitive to tryptic digestion than free EPSPS (compare Figures 3 and 7), indicating higher accessibility of the MurA trypsin cleavage sites. This in turn may be due to a higher flexibility and a more open conformation of MurA as compared to EPSPS. In fact, the size of the two substrates is very different, and on the basis of the assumption that the much bulkier UDP-*N*-acetylglucosamine requires a larger point of entry into the binding pocket, MurA would be expected to exhibit a higher degree of flexibility. Comparison of the crystallographic 3D-structures of uncomplexed MurA and EPSPS indicates a more open conformation of the latter opposite to the results obtained from limited proteolysis. However, the larger separation of the domains in EPSPS has been proposed to be the result of ammonium sulfate induced crystal packing forces (*28*).

In both enzymes, the most extensive protection against proteolysis was exerted by binding of both the enolpyruvyl-accepting substrate (UDPNAG and S3P, respectively) and an inhibitor (fosfomycin and glyphosate, respectively). This finding is quite intriguing since these inhibitors do not share a common mode of action: fosfomycin inhibits MurA by forming a covalent adduct with the thiol group of cysteine 115, whereas glyphosate forms a noncovalent complex with EPSPS. Furthermore, unlike fosfomycin, glyphosate does not compete for the substrate binding sites and, as recently proposed, affects EPSPS activity allosterically (*11*). Despite these crucial differences in binding mode and location, binding of the inhibitor in conjunction with the enolpyruvyl-accepting substrate appears to lock the protein in a tightly packed conformation. For MurA, this complex was described as the closed conformation which is stabilized by a network of interactions between fosfomycin, UDPNAG, and the enzyme (*10*). In the absence of any high-resolution structure of the putative closed conformation of EPSPS, it is not possible to describe any interactions at the molecular level. However, by analogy to MurA, it appears likely for EPSPS that glyphosate similarly stabilizes the closed conformation by comparable interactions to S3P and the protein. Recent efforts to identify the glyphosate-binding site provided some evidence for the involvement of a critical region around amino acids 90−102 (*29*, *30*). Within this region, single amino acid replacements were found to confer glyphosate tolerance, i.e., G96A (*30*) and P101S (*31*, *32*). In Figure 10, those three peptides (amino acids 123−134, 140−152, and 355−367, respectively) which are released by trypsin cleavage in unliganded EPSPS are highlighted in the structure of EPSPS (right structure in Figure 10). These regions in the upper and lower domains of the enyzme are protected by binding of both S3P and glyphosate. Assuming that a conformational change in EPSPS, similar to MurA, occurs, it is conceivable that glyphosate binds to the proposed region (90−102) which might undergo the most distinct conformational change, analogous to the region in MurA. On the other hand, the location of the protected areas shown in Figure 10 (right structure) visualizes that the third shielded trypsin cleavage site is far apart and that the conformational

change presumably affects the entire protein and not just the immediate vicinity of the glyphosate-binding site. A point in case is histidine 385: this residue was reported earlier to be located at or near the glyphosate/PEP binding site in EPSPS (*33*−*35*). However, as shown in Figure 10, histidine 385 is not part of any of the fragments found to be protected by ligand binding.

The impact and extent of the conformational change on the accessibility of tryptic cleavage sites is also emphasized by our studies on MurA. It was shown that free MurA is quite susceptible to tryptic digestion giving rise to a multitude of peptide fragments. In the presence of UDPNAG and fosfomycin, however, most of the cleavage sites in the protein are no longer readily accessible. This clearly demonstrates that the protection of tryptic cleavage sites is not restricted to areas adjacent to the substrate- and inhibitor-binding sites.

In the present study, we have employed three independent methods to monitor the time course of tryptic digestion of MurA and EPSPS: analysis by means of SDS−PAGE, enzymatic activity measurements, and MALDI mass spectrometry. Each of the methods was found to provide valuable information concerning the effect of substrates and inhibitors on the proteolytic susceptibility of the two proteins investigated. Following the decrease in activity in the time course of digestion gives information on the global proteolytic susceptibility under the incubation conditions. SDS−PAGE visualizes large proteolytic fragments. When the progress of proteolysis of MurA was followed by SDS−PAGE, the sample was denatured by addition of SDS and heat treatment prior to loading on the gel. This procedure, however, results in the rapid unfolding of the protein, and it was found that some residual trypsin activity was still present in the sample, resulting in further trypsinolysis of the unfolded proteins. As a consequence, the protective effect of ligands on MurA was generally underestimated by this method. Comparison of Figures 1 and 2 may serve as a point in case: while addition of UDPNAG and PEP fully protect MurA activity as shown by activity measurements (Figure 2), SDS−PAGE suggests substantial, but not complete, protection of the protein (Figure 1). Apart from the underestimation of the protective effect of ligands, the higher digestibility of MurA was confirmed by MALDI mass spectrometry. Thus, the conclusion that EPSPS is more stable toward proteolysis than MurA is well substantiated. As far as the activity assays are concerned, tryptic digestion of MurA may not necessarily diminish enzyme activity as long as cleavage of catalytically essential peptides of the protein does not occur and hence may proceed without being detected by an activity assay. Although analysis of tryptic digests by nondenaturing gel electrophoresis provided no evidence for the existence of catalytically active MurA fragments, MALDI mass spectrometry revealed small peptides being released in the presence of UDPNAG and PEP, i.e., under conditions where no loss of enzymatic activity could be observed in the assay.

In summary, MALDI mass spectrometry proved to be a convenient and fast method to gain site-specific information of regions susceptible to proteolytic degradation and to study the protective effects of ligands. The latter application may also be exploited in the evaluation and development of new protein inhibitors.

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