Substrate Deactivation of Phenylalanine-Sensitive 3-Deoxy-D-*arabino*-heptulosonate 7-Phosphate Synthase by Erythrose 4-Phosphate†

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ABSTRACT: 3-Deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (DAH7PS, EC 4.1.2.15) catalyzes the condensation of phosphoenolpyruvate (PEP) with erythrose 4-phosphate (E4P) to give DAH7P via an ordered sequential mechanism. In the absence of PEP (the first substrate to bind), E4P binds covalently to the phenylalanine-sensitive DAH7PS of *Escherichia coli*, DAH7PS(Phe), deactivating the enzyme. Activity is restored on addition of excess PEP but not if deactivation was carried out in the presence of sodium cyanoborohydride. Electrospray mass spectrometry indicates that a single E4P is bound to the protein. These data are consistent with a slow, reversible Schiff base reaction of the aldehydic functionality of E4P with a buried lysine. Molecular modeling indicates that Lys186, a residue at the base of the substrate-binding cavity involved in hydrogen bonding with PEP, is well placed to react with E4P forming an imine linkage that is substantially protected from solvent water.

The enzyme 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (DAH7PS,[1] EC 4.1.2.15) catalyzes the condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) to give 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAH7P) (*1*, *2*). This reaction is the first committed step of the shikimate pathway (*3*−*6*), the biosynthetic pathway to the aromatic amino acids in plants and microorganisms. The enzymology of this pathway continues to attract attention because the enzymes are potential targets for herbicides (*7*−*9*) and for antiparasitic (*10*−*12*) and antimicrobial agents (*13*). Many species have more than one isozyme of DAH7PS (*14*−*16*). These isozymes are distinguished by their feedback sensitivity to phenylalanine, tyrosine, or tryptophan (*17*−*19*), the final products of the pathway (Scheme 1).

The aldol-like condensation between the olefinic PEP and the aldehydic E4P requires a divalent metal ion (e.g., Mn^{2+}) (*20*−*23*), and, during the course of the reaction, breakage of the carbon−oxygen bond of PEP results in the direct release of the other reaction product, phosphate (*24*, *25*). The reaction follows an ordered sequential mechanism, where PEP is the first substrate to bind and DAH7P is the last product to leave (*16*, *18*, *26*, *27*). The reaction has also been shown to proceed with a defined stereochemical course with respect to C3 of PEP, with the *si* face of PEP attacking the *re* face of E4P to give products DAH7P and phosphate (*28*, *29*). These findings require that at some stage in the DAH7PS reaction a nucleophile must attack the central carbon of PEP. There are three possible candidates for the nucleophile. First, the nucleophile may simply be water as has been proposed (*30*), giving acyclic DAH7P after phosphate elimination. DAH7P then cyclizes to the hemiacetal form of DAH7P shown in Scheme 1. Second, the C3 hydroxyl of cosubstrate E4P may act as the initial nucleophile to give a cyclic intermediate, with loss of phosphate and the attack of water to form cyclic DAH7P. Third, an enzyme-based nucleophile may attack C2 of PEP, with its ultimate substitution by water prior to release of products.

Despite recent publications describing the structures of the phenylalanine-sensitive DAH7PS, DAH7PS(Phe), from *Escherichia coli* with bound Pb^{2+} and PEP (*30*) and with Mn^{2+} and bound 2-phosphoglycolate (PGL) (*31*), the structure of 3-deoxy-D-*manno*-octulosonate 8-phosphate synthase (KDO8PS), for which the cosubstrate of PEP is arabinose 5-phosphate (A5P) (*32*, *33*), and the substrate specificity of DAH7PS(Phe) (*34*, *35*), there no clear understanding at the atomic level of the mechanism of the DAH7PS(Phe) reaction and the roles of various conserved residues in the active sites of either DAH7PS(Phe) or KDO8PS. The distances recorded between the two phosphate-binding sites in these structures, however, suggest that the C3 hydroxyl of E4P (or of A5P in KDO8PS) cannot act as a nucleophile in the enzymatic reaction (*32*−*35*).

We report here the interesting result that, in the absence of PEP, the cosubstrate E4P deactivates the DAH7PS(Phe) from *E. coli* by forming a covalent bond with the enzyme, most likely with a lysine moiety in the active site. These results explain the apparent instability of DAH7PS(Phe) in the presence of excess E4P that had been reported by several

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[1] Abbreviations: DAH7P, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; DAH7PS(Phe), phenylalanine-sensitive 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase; KDO8PS, 3-deoxy-D-*manno*-octulosonate 8-phosphate synthase; E4P, erythrose 4-phosphate; PEP, phosphoenolpyruvate; A5P, arabinose 5-phosphate; G3P, glyceraldehyde 3-phosphate; R5P, ribose 5-phosphate; PGL, 2-phosphoglycolate; BTP, 1,3-bis(tris(hydroxymethyl)methylamino)propane; ES-MS, electrospray mass spectroscopy.

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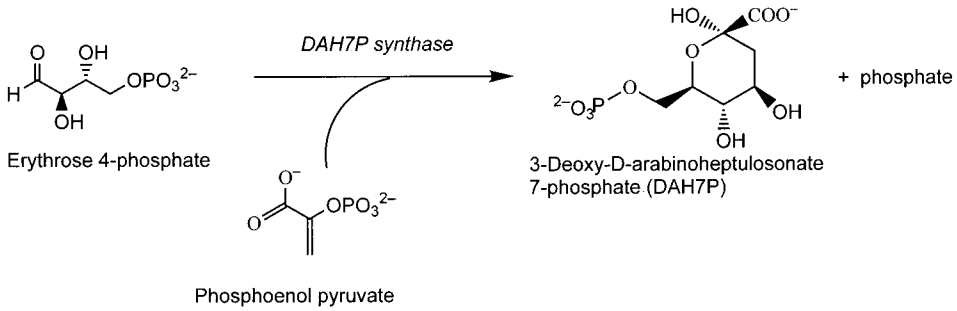
Scheme 1: Shikimate Pathway



groups (*17*) and give additional insight into the metal-catalyzed inactivation of DAH7PS(Phe) (*36*).

MATERIALS AND METHODS

Preparation and Handling of E4P and DAH7PS(Phe). E4P is a notoriously difficult compound with which to work. It is unstable in solution, being susceptible to elimination and hydrolysis (*37*, *38*). In concentrated solutions it exists in a dimeric form, which converts slowly to the monomeric species on dilution (*39*). It has also been reported to form imines readily (*40*), rendering it unsuitable for study in buffers containing primary amines. Thus, E4P (Sigma) solutions were left for a minimum of 2 h before use in order for the dimer to convert to monomer.

PEP (Sigma) and E4P solutions were prepared by dissolving a weighed amount of each material in a measured volume of BTP buffer (Sigma, 50 mM, pH 6.8). Accurate concentrations were determined by following the loss of PEP at 232 nm ($\epsilon = 2.8 \times 10^3$ M^{-1} cm^{-1}) in the DAH7PS(Phe) reaction on complete conversion of one of the substrates (with the other in excess) using a Varian Cary 1E UV spectrophotometer. Alternatively, E4P concentrations were determined by monitoring the oxidation of NADH at 340 nm ($\epsilon = 6.2 \times 10^3$ M^{-1} cm^{-1}) under the action of glycerol 3-phosphate dehydrogenase, following conversion of E4P to glycerol 3-phosphate by the action of transaldolase and triose phosphate isomerase (*41*).

The expression and purification of recombinant DAH7PS-(Phe) followed the procedure of Stephens and Bauerle (*21*), with the following modifications to ensure reproducible metal ion content. ApoDAH7PS(Phe) was prepared by dialysis against a 10 mM BTP solution containing 200 μM PEP and 1 mM EDTA. After 18 h the enzyme was dialyzed against two changes (12 h) of a 10 mM BTP solution containing 200 mM PEP and 10 mM EDTA. ApoDAH7PS was frozen in 100 μL aliquots in liquid nitrogen and stored at -70 °C. Protein concentrations were determined by Bradford assay (Bio-Rad) using BSA as a standard. Values obtained were corrected by a factor of 0.65 according to Stephens and Bauerle (*21*). This correction factor was confirmed by comparison with results from amino acid analysis.

Metal ions were removed from buffer and reagent solutions by treatment with Chelex 100 resin (Bio-Rad). Solutions were buffered at pH 6.8 with 50 mM BTP and were filtered through a 0.45 μm membrane prior to use. Protein solutions contained 10−20 mM PEP to stabilize DAH7PS(Phe) and 10 μM EDTA to sequester residual metal ions. The standard assay involved the addition of ~0.05 unit of enzyme to a solution containing PEP (150 μM), E4P (350 μM), and $MnSO_4$ (50 μM) in 50 mM BTP buffer at pH 6.8 at 25 °C. Binding of Mn^{2+} to the apoenzyme takes place on a faster time scale than does reaction of PEP with E4P. Activity (rate of consumption of PEP) was monitored at 232 nm ($\epsilon = 2.8 \times 10^3$ M^{-1} cm^{-1}) at 25 °C (*21*).

Deactivation Studies. All deactivation studies were performed at pH 6.8 in 50 mM BTP buffer at 22 °C unless otherwise noted. Each run involved addition of protein solution (concentrations in the range 8−15 μM) to solutions containing different concentrations of E4P (0.19−1 mM), sodium cyanoborohydride (0, 5−7 mM), Mn^{2+} (0, 50 μM), glyceraldehyde 3-phosphate (G3P, Sigma, 1 mM), and ribose 5-phosphate (R5P, Sigma, 3 mM). The experiments that examined the protection 2-phosphoglycolate (PGL, Sigma) affords against deactivation of DAH7PS by E4P were performed at 12 mM DAH7PS with an E4P concentration of 810 mM at a temperature of 18 °C. PGL was present in a concentration of either 1 or 5 mM.

Aliquots (~0.05 unit of enzyme) were taken at various times, and their activity was measured in the standard assay system as detailed above and compared against the control solution, which lacked only the E4P.

Electrospray Mass Spectrometry. Following deactivation protein samples were gel-filtered (PD-10 column, Amersham Pharmacia). As noted, some samples were then divided in two at this stage with half of the sample treated with $NaCNBH_3$ (5 min, 12 mM) before passage through another PD-10 column. By means of Centricon 10 microconcentrators (Amicon), all protein solutions were then concentrated and desalted to a protein concentration of ~100 μM in 4 mM NH_4HCO_3 (pH 8) before dilution to a 50:50 (v/v) acetonitrile−water mixture acidified to 1% acetic acid. Aliquots of 10 μL were injected into the mass spectrometer (VG BioQ quadrupole mass spectrometer in positive ion mode contolled by Micromass MassLynx software, calibrated to myoglobin). Raw *m*/*z* data were collected. Raw spectra were transformed using MassLynx deconvolution software from *m*/*z* peaks with charges ranging between +22 and +38.

Molecular Modeling. TURBO (*43*) was used to orient and position E4P in the PEP-binding pocket and to examine models for plausible stereochemistry. Conformations of active site residues were left unaltered, except for reorientation of Lys97 [remaining within the same allowed rotamer conformation that was observed in the X-ray structure of DAH7PS(Phe)] to optimize hydrogen bonding with E4P (*30*).

RESULTS

Activity of DAH7PS(Phe). Our values for K_M(PEP), K_M(E4P) (at a PEP concentration of 150 mM), and k_{cat} are

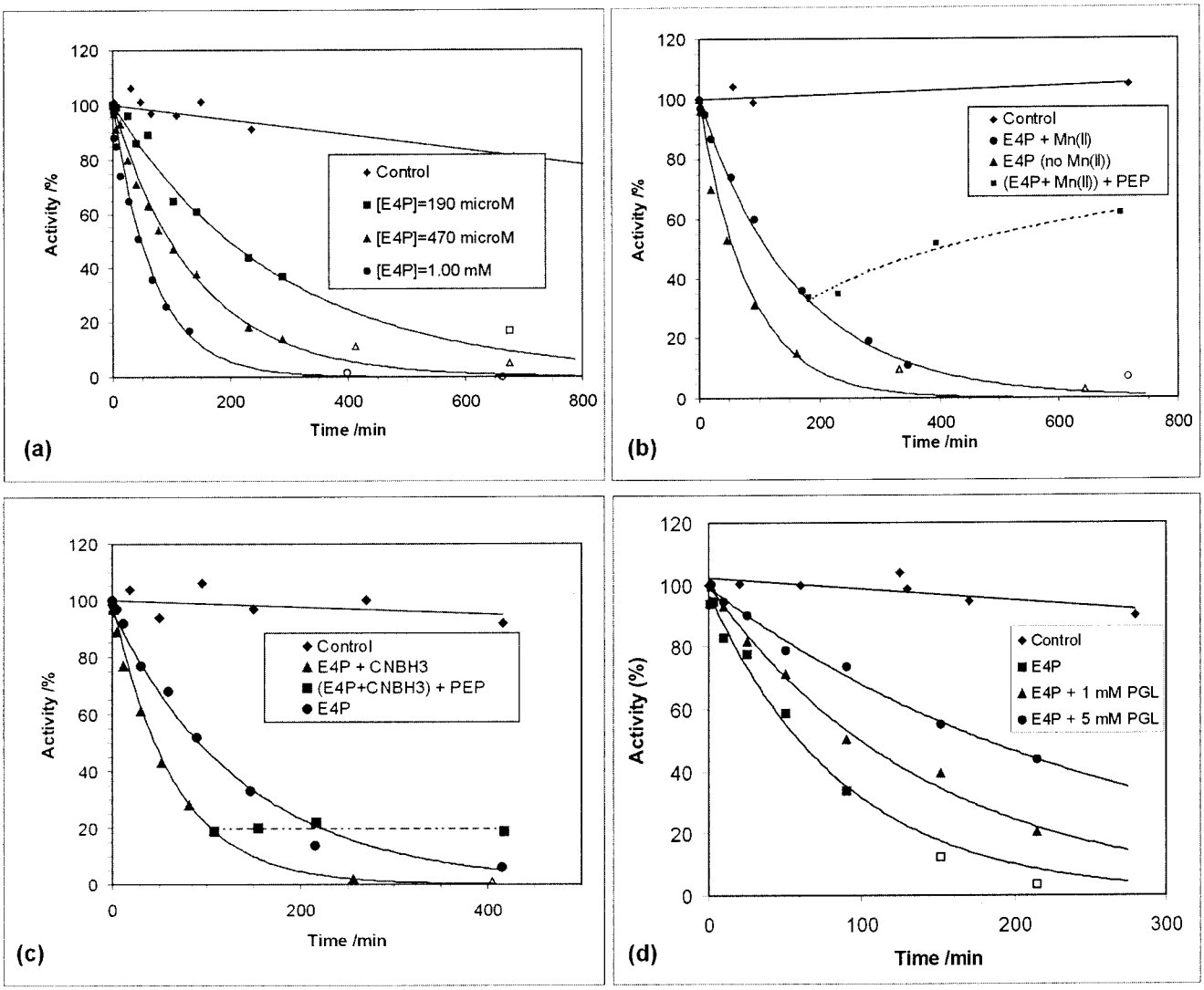


FIGURE 1: Deactivation of DAH7PS(Phe) by E4P. The decrease in activity to ~25% of the original activity is well fit (correlation coefficients > 0.98) by a simple first-order process. Data included in fitting a single exponential decay are shown as filled symbols; data not included are shown as open symbols. (a) Concentration dependence of the rate of deactivation ([DAH7PS(Phe)] = 8 μM; [Mn^{2+}] = 50 μM). Concentrations of E4P are indicated on the diagram. (b) Reactivation of DAH7PS(Phe). When activity of DAH7PS(Phe) had decreased to 35% of the original activity, PEP (650 μM) was added to one portion of the reaction mixture (originally [DAH7PS(Phe)] = 8 μM; [E4P] = 320 μM; [Mn^{2+}] = 50 μM), partly restoring activity. In the absence of Mn^{2+} deactivation occurs more quickly. (c) Deactivation in the presence of sodium cyanoborohydride is irreversible and occurs more quickly than in its absence. In the presence of 7 mM $NaCNBH_3$, addition of PEP (560 μM) and Mn^{2+} (60 μM) failed to restore activity to the reaction mixture (originally [DAH7PS(Phe)] = 9 μM; [E4P] = 450 μM). (d) Deactivation by E4P is slowed in the presence of 2-phosphoglycolate ([DAH7PS(Phe)] = 12 μM; [E4P] = 810 μM; [2-phosphoglycolate] = 0, 1, or 5 mM, 18 °C).

respectively 2.0 mM, 21 mM, and 71 s^{-1}, values which with one exception are very similar to those reported by Stephens and Bauerle (*21*). They report values of 2.0 μM, 150 μM, and 61 s^{-1}, respectively. K_M(E4P) is very sensitive to metal ion, and this may be the source of the discrepancy between our value for K_M(E4P) and that of Stephens and Bauerle (*21*).

Deactivation of DAH7PS(Phe) by E4P. Addition of excess amounts of E4P (190, 470, and 1000 mM) to DAH7PS(Phe) led to a concentration-dependent reduction in enzyme activity, with times of 205, 100, and 40 min, respectively, for activity to be reduced to half that of the control (Figure 1a). This deactivation was substantially, but not completely, reversed by the addition of PEP in excess of the amount of E4P initially added (Figure 1b). The presence of sodium cyanoborohydride doubled the rate of deactivation, with negligible effect (<5% deactivation over 400 min) on the activity of the control (Figure 1c). Moreover, activity could not be restored on addition of PEP (Figure 1c). Incubation of DAH7PS(Phe) with G3P, which, in the presence of PEP, is not a substrate of the enzyme, led to deactivation of the enzyme, but only in the presence of sodium cyanoborohydride. On the other hand, R5P, which, in the presence of PEP, is a (poor) substrate of the enzyme (*35*), showed only competitive inhibition (K_i > 6 mM). PGL, which has been shown crystallographically to bind at the PEP-binding site of DAH7PS(Phe) (*31*), protects the enzyme against deactivation by E4P (Figure 1d). The protection was observed to depend on the concentration of PGL employed. We have estimated the K_i of PGL to be in the order of 45 ± 15 μM.

In the presence of Mn^{2+}, deactivation of DAH7PS(Phe) by E4P is substantially retarded (Figure 1b), a result that complements the observation that redox-active metals ions, such as Cu^{2+} and Fe^{2+} (but not Mn^{2+}), accelerate deactivation of the enzyme by catalyzing the formation of a disulfide

Table 1: Relative Rates of Deactivation of DAH7PS(Phe) under Different Conditions

conditions[a]	relative rate
E4P	1
E4P + Mn^{2+}	0.5
E4P + $CNBH_3$	2.1
E4P + PGL (1 mM)	0.6
E4P + PGL (5 mM)	0.3
G3P (1)	no detectable deactivation
G3P (1) + $CNBH_3$	~0.2
R5P (2)	no detectable deactivation

[a] All values are given relative to the rate of deactivation of DAH7PS(Phe) at the same concentrations of DAH7PS(Phe) and E4P and at the same temperature, except (1) where the G3P concentration was 1 mM relative to an E4P concentration of 400 mM and (2) where the R5P concentration was 3 mM relative to an E4P concentration of 1 mM. Concentrations of Mn^{2+} and $NaCNBH_3$ were 50 μM and 7 mM, respectively, when used.

bridge between metal−ligand Cys61 and nearby Cys328 (*34*). The relative rates of deactivation of DAH7PS are summarized in Table 1. Rates are recorded relative to the rate of deactivation with E4P alone to account for differences between experiments in the temperature and the concentrations of enzyme and E4P employed and for the variations that occurred between different enzyme preparations. Relative rates are consistent to 10% between duplicate experiments.

Electrospray Mass Spectrometry of DAH7PS(Phe)−E4P. The mass spectra of controls showed a single peak for unmodified DAH7PS(Phe) in the range 38011−38014 Da, with an estimated standard deviation of ±5 Da for the mass of DAH7PS(Phe) in each spectrum. The calculated mass based upon the protein sequence plus the N-terminal methionine is 38009 (*44*). After incubation with E4P to 30% of the original activity a sample was subjected to gel filtration to remove of excess E4P and split into two portions. One portion was immediately prepared for and subjected to ES-MS and showed a product peak (Figure 2a, Table 2) of mass 179 Da greater than that of the control, corresponding to the condensation of protein with E4P (198 Da) accompanied by the loss of water (18 Da). Immediate treatment of the second portion with sodium cyanoborohydride, followed by gel filtration to remove excess reductant, showed a single product peak of mass 182 greater than that of the unmodified protein (Figure 2b, Table 2). The *unlikelihood* of the single but heterogeneous DAH7PS(Phe)−E4P adduct is addressed in the Discussion. On extended incubation to <5% of the original activity, a peak at 38046 developed in the presence of Mn^{2+} (Table 2). The ES-MS for a sample injected just after mixing of DAH7PS(Phe) with E4P revealed the presence of a product with mass 183 Da greater than that of the unmodified protein, although the spectrum is of lesser quality because of the background ions. This result establishes that the protocol for preparing low-salt solutions for optimal ES-MS does not cause covalent modification of DAH7PS(Phe).

When DAH7PS(Phe) is incubated with E4P in the presence of sodium cyanoborohydride, a covalent species is formed, again with a mass difference between modified and unmodified protein of 182 (Figure 3a, Table 2). On extended incubation (>95% deactivation) in the presence of sodium cyanoborohydride, predominantly doubly and triply modified species are found, along with a small amount of quadruply

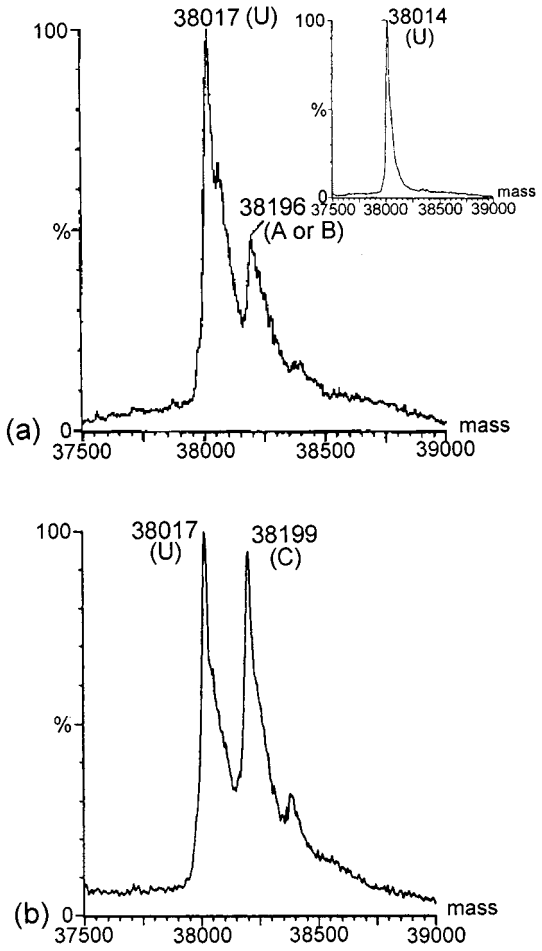


FIGURE 2: Electrospray mass spectrometry of DAH7PS(Phe) partly deactivated by E4P. (a) ES-MS of DAH7PS(Phe) at 30% of the original activity, showing the parent peak and condensation product of DAH7PS(Phe) and E4P. The sample for ES-MS was gel-filtered and prepared for ES-MS, as described in Materials and Methods. The insert shows the ES-MS of the unmodified protein of the control. Initial conditions: [DAH7PS(Phe)] = 9 μM; [E4P] = 320 μM. (b) As for (a), except that the sample was treated with 12 mM $NaCNBH_3$ for 5 min prior to gel filtration and preparation for ES-MS.

modified species (Figure 3b, Table 1). The activity of the control, which contained sodium cyanoborohydride, showed less than 5% decrease over the course of the experiment (500 min).

Molecular Modeling of the DAH7PS(Phe)-E4P Species. As apoDAH7PS is deactivated by E4P, and Mn^{2+} appears to retard deactivation by E4P, we chose to model using structures without metal in the active site. The active site of DAH7PS with PEP bound is shown in the stereo diagram of Figure 4a. The right-most frame of Figure 4a is a space-filling representation of the active site devoid of substrate and metal ion. E4P was linked to DAH7PS(Phe) via imine linkages to active site lysines, Lys186 or Lys97. Attached to Lys186, E4P in an extended conformation is well positioned to hydrogen bond to other active site residues, with minimal adjustment of side chains to achieve optimal hydrogen bonding, utilizing the hydrogen-bonding capacity of both the protein side chains and of E4P itself (Figure 4b). The hydrogen bond shown between the carboxylate group of active site residue Asp326 and a terminal phosphate oxygen atom is acceptable, as at the pH of the deactivation

Table 2: Mass Spectrometry of E4P−DAH7PS(Phe) Reactions

incubation conditions (all performed at 50 μM Mn^{2+})	% deactivation	postincubation treatment	observed masses (Da)	
			control	incubated sample
[E4P] = 320 μM, [DAH7PS(Phe)] = 9 μM, 22 °C, t = 150 min	>90%	gel filtration	38014 (U) (Figure 2a inset)	38017 (U), 38196 (A or B) (Figure 2a)
[E4P] = 320 μM, [DAH7PS(Phe)] = 9 μM, 22 °C, t = 150 min	>90%	gel filtration, $NaCNBH_3$, gel filtration	38014 (U) (Figure 2a inset)	38017 (U), 38198 (B or C) (Figure 2b)
[E4P] = 400 μM, [DAH7PS(Phe)] = 90 μM, 22 °C, t = 20 min	not recorded	none	38011 (U)	38017 (U), 38200 (A or C)
[E4P] = 450 μM, [DAH7PS(Phe)] = 9 μM, $NaCNBH_3$, 22 °C, t = 48 min	50%	gel filtration	38014 (U)	38021 (U), 38206 (C), 38403 (D) (Figure 3a)
[E4P] = 450 μM, [DAH7PS(Phe)] = 9 μM, $NaCNBH_3$, 22 °C, t = 260 min	>95%		38014 (U)	38224 (C), 38395 (D), 38570 (E), 38749 (F) (Figure 3b)
[E4P] = 320 μM, [DAH7PS(Phe)] = 8 μM, 32 °C, t = 350 min	>95%	gel filtration, $NaCNBH_3$, gel filtration	38012 (U)	38046 (H), 38199 (A or B)

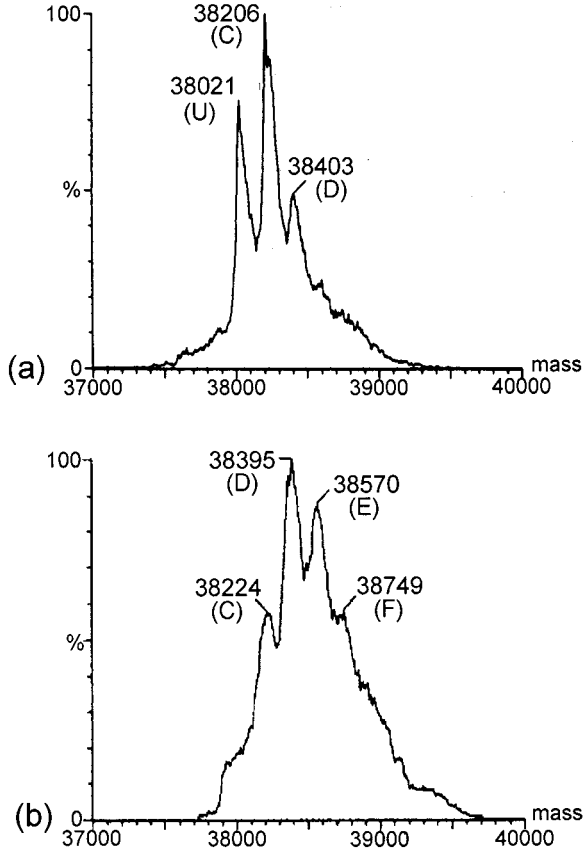


FIGURE 3: Deactivation of DAH7PS(Phe) by E4P in the presence of 5 mM $NaCNBH_3$. (a) ES-MS of DAH7PS(Phe) at 50% deactivation shows the parent ion and singly and doubly modified ions. (b) ES-MS of DAH7PS(Phe) at 95% deactivation showing singly, doubly, triply, and quadruply modified species. Initial deactivation conditions: [DAH7PS(Phe)] = 9 μM; [E4P] = 450 mM; 22 °C.

studies, the phosphate group is expected to be singly protonated, especially as the phosphate group is substantially exposed to the solvent (Figure 4b). Attached to Lys97, E4P in an extended conformation could not be oriented to utilize the hydrogen-bonding capacity of the protein side chains without substantial reorganization of side-chain conformations. In a less favorable cisoid conformation, E4P can be linked to Lys97 with its phosphate group occupying the same site as the phosphate group of PEP (Figure 4c). The space-filling representation shows that the E4P-Lys97 imine bond is substantially exposed to the solvent (Figure 4f), in contrast to the E4P−Lys186 imine bond, where it is completely buried (Figure 4b). G3P and R5P do not enjoy the same stabilizing contacts as E4P does when linked to Lys186.

DISCUSSION

A Single Lysine at the Active Site Is Modified by E4P. The mass difference of ~180 Da between unmodified protein and protein modified by incubation with E4P is consistent with a condensation reaction in which E4P becomes covalently attached to DAHP7S(Phe) (with loss of water). Several lines of evidence indicate that the aldehydic E4P forms a Schiff base, which affects enzyme activity, with the amino function of an active site lysine: (i) in the presence of sodium cyanoborohydride, the rate of deactivation of the enzyme is increased, consistent with irreversible reduction of a labile Schiff base adduct to a stable amine; (ii), in part a corollary of (i), multiple addition of E4P occurs only in the presence cyanoborohydride; (iii) R5P, which differs from E4P by an additional −CH(OH)− moiety, causes only weak competitive inhibition, indicating that it is unable to access the lysine that E4P accesses; (iv) G3P causes deactivation only in the presence of sodium cyanoborohydride, which reduces a labile imine moiety to a stable amine moiety; and (v) PGL, which has been shown crystallographically to bind noncovalently in the active site of DAH7PS(Phe) (*31*), protects against the deactivation of the protein by E4P.

The persistence of a singly modified protein through gel filtration and removal of excess E4P indicates that one E4P−imine bond is substantially stabilized with respect to all other such bonds, either because the imine bond is buried (kinetic stabilization) and solvent inaccessible and/or because of strong nonbonded interactions of E4P with the protein (thermodynamic stabilization). Further evidence for a single reactive lysine is found in the observation that, at 50% deactivation of the protein in the presence of sodium cyanoborohydride, the predominant species has only a single modification. Only when almost all activity has been lost are multiply modified species observed by mass spectrometry. Even so, the deactivation follows a simple exponential in general to more than 80% deactivation (Figure 1), indicating that modification of the enzyme at other sites has negligible effect on activity. Furthermore, G3P causes deactivation only when sodium cyanoborohydride is present, indicating that the imine bond formed by this less bulky aldehyde is not stabilized relative to all other Lys−G3P linkages.

The recently published structure of DAH7PS(Phe) (*30*) allows this reactive lysine to be tentatively identified. Only

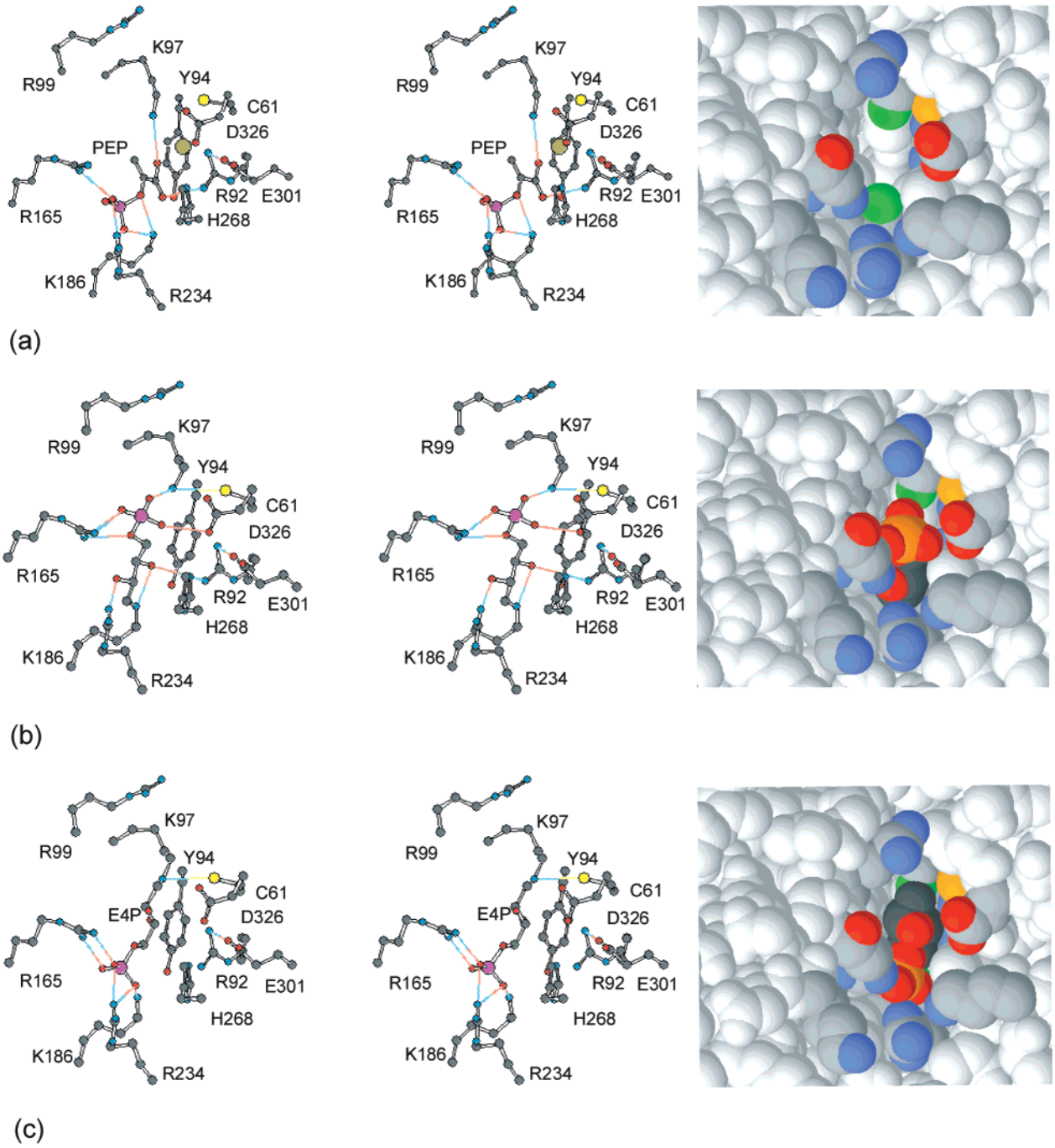
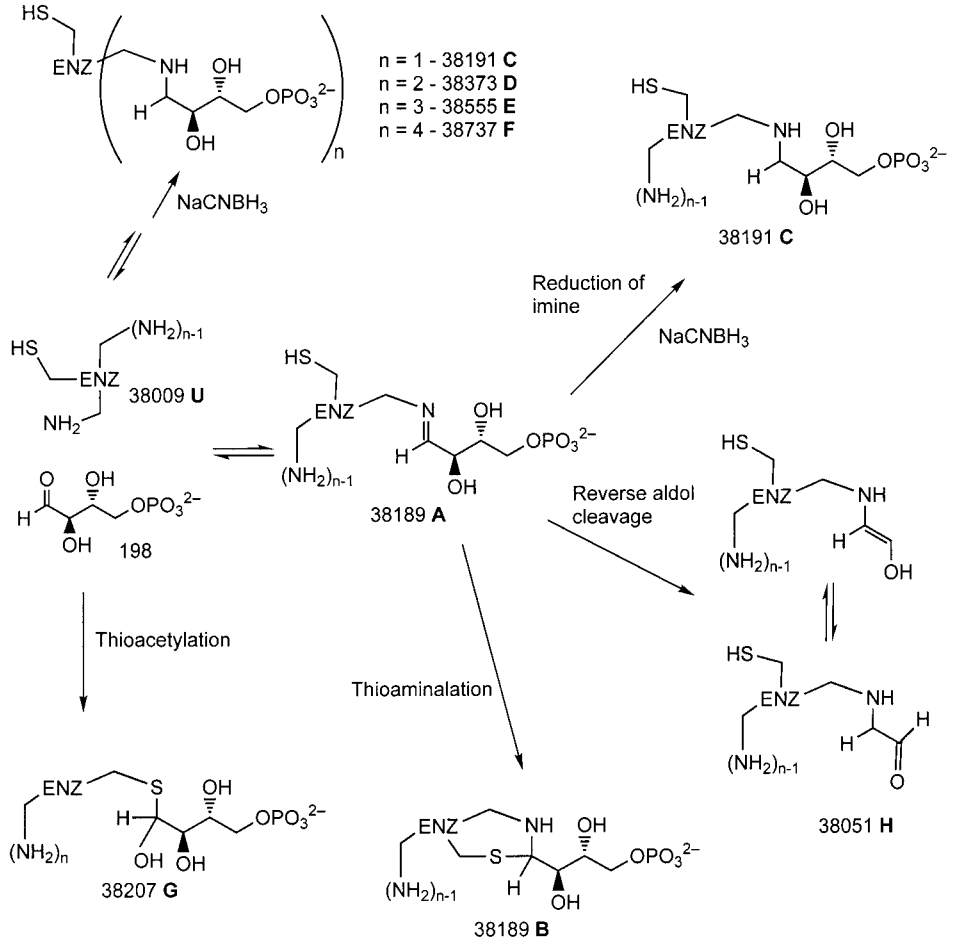


FIGURE 4: Active site of DAH7PS(Phe) and possible DAH7PS(Phe)−E4P species. Coordinates for the protein and PEP are taken from the structure by Shumilin et al. (*21*) (PDB ID: 1QR7). (a) Stereo diagram of PEP-binding site with PEP bound (*21*). The right-most frame is a space-filling representation of the substrate-binding pocket (PEP and E4P omitted), showing solvent exposure of lysine and arginine residues (CPK colored except for NZ of Lys). (b) Stereo diagram of a putative Lys186−E4P moiety. The right-most frame is a space-filling representation of the putative Lys186−E4P moiety, showing burial of Lys186. (c) Stereo diagram of a putative Lys97−E4P moiety. The right-most frame is a space-filling representation of the putative Lys97−E4P moiety, showing partial exposure of the imine linkage.

two lysines, Lys97 and Lys186, are sufficiently close to the active site to effect deactivation when modified. These lysines are structurally equivalent to Lys60 and Lys138 in the structure of KDO8PS (*32*, *33*). For DAH7PS(Phe), Lys186 lies at the base of the PEP-binding pocket and, when attached to E4P, is almost completely buried (Figure 4b). Molecular modeling of the DAH7PS(Phe)−Lys186−E4P species indicates not only that E4P fits into the PEP-binding site but also that its hydroxyl and phosphate moieties engage in favorable hydrogen-bonding interactions with residues of the PEP and presumed E4P binding site (Figure 4b). Moreover, in the near absence of PEP [the micromolar residual concentration of PEP is on the order of its dissociation constant from DAH7PS(Phe)] and with the buried residues (Lys186, Arg92, and Arg234) in close proximity, we infer that in the unmodified protein Lys186 is neutral (and hence reactive) in the absence of negatively charged ions to stabilize the cluster of otherwise positively charged residues.

On the other hand, Lys97, even when attached to E4P, remains substantially exposed to bulk solvent (Figure 4c). Molecular modeling places the phosphate moiety of the DAH7PS(Phe)−K97−E4P species at the phosphate-binding

Scheme 2: Reactions of E4P with Lysine and Cysteine Residues[a]



[a] The letters are used to identify species in Figures 2 and 3.

site for PEP. The hydroxyl moieties of E4P do not interact directly with any protein residues, but indirect interactions mediated by water molecules are possible. There is nothing remarkable about the environment of the terminal amine moiety of Lys97 to suggest that it is more active than other surface-exposed lysines. Thus, we favor Lys186 as the reactive lysine, but unequivocal identification of the site of E4P attachment must await mass spectral analysis of tryptic digests of the DAH7PS(Phe)−E4P species.

Additional E4P-Induced Modifications of DAH7PS(Phe). Although addition of excess PEP to partly deactivated enzyme restores activity, activity is restored, at best, to 60% of that of the control (Figure 1b). ES-MS of the product of deactivations performed at elevated temperatures may provide some clues into the species that are associated with this irreversible deactivation. ES-MS of a reaction mixture incubated at 32 °C to less than 5% of original activity in the presence of Mn^{2+} and in the absence of sodium cyanoborohydride shows a peak at 38046 Da, consistent with the product (of expected mass of 38050 Da; species H in Scheme 2) resulting from a retroaldol elimination of ethene 2-phosphate from the DAH7PS(Phe)−E4P imine species (A, Scheme 2). It is also noted that Cys61 which is located near Lys97 is well positioned (see Figure 4c) for nucleophilic attack, either on the aldehyde directly to give a thioacetal (G, Scheme 2) or on the imine bond to give a thioaminal species (B, Scheme 2). Mass spectral analysis is inconsistent with an E4P−enzyme adduct that is a result of a direct interaction of a reactive cysteine and the E4P aldehydic functionality (G, Scheme 2) but cannot distinquish between a thioaminal derivative (B, Scheme 2) and the imine DAH7PS(Phe)−E4P species (A, Scheme 2) as their predicted masses are identical.

E4P Deactivation of Other Enzymes. E4P has been reported to deactivate several other enzymes, including rabbit muscle aldolase and aspartate aminotransferase. In the case of E4P deactivation of rabbit muscle aldolase (fructose 1,6-biphosphate aldolase) (*45*, *46*), cysteine was initially implicated in the formation of a thioaminal species (*47*), a formation possible only in the absence of the cosubstrate dihydroxyacetone phosphate. However, subsequent studies on chemically modified protein (*48*) and on site-directed mutants (*49*, *50*) indicated that modification of cysteine was not the cause of enzyme deactivation but that lysine was. An active site lysine is now implicated in the mechanism of deactivation (*51*). Analogous to DAH7PS(Phe), deactivation of aspartate aminotransferase by E4P (and G3P) could be reversed only in the absence of, in this case, sodium borohydride (*52*, *53*). Again an active site lysine was implicated in the mechanism of deactivation. For the reaction of E4P with DAH7PS(Phe), mass spectrometry provides hard evidence that deactivation is primarily caused by condensation of E4P with a single reactive lysine of the active site.

In Vivo Implications of Substrate Deactivation. It seems strange that DAHP7S(Phe) should be deactivated by exposure to a compound that its very role demands it to come in contact with. However, for DAHP7S(Phe), no deactivation by substrate E4P occurs in the presence of PEP. PEP binds

to the apoenzyme with at least 100 times greater affinity than does E4P, and, moreover, the cellular concentration of PEP is substantially greater than that of E4P. Thus, there is essentially no free enzyme in vivo, and it is only in vitro that PEP can be stripped from the active site by reaction with excess E4P to produce the free enzyme that is then deactivated by reaction with E4P. It therefore appears that DAH7PS(Phe) has a reactive functional group, essential for catalysis and possibly the same group as that to which E4P attaches, that it protects from reactive aldehydic second substrates, such as E4P, by the prior binding of PEP. Thus, the reaction is poised to go down the correct path only when E4P is introduced to the DAH7PS(Phe)−PEP complex.

CONCLUSIONS

E4P forms a metastable Schiff base linkage with a lysine residue on DAH7PS resulting in deactivation of the enzyme. We propose that this residue is either Lys186 or Lys97, two key residues of the active site of DAH7PS(Phe), the former lysine being part of the PEP's phosphate-binding site and the latter being part of the presumed binding site of the phosphate group on E4P. Further slow reactions lead to irreversible deactivation of DAH7PS(Phe) by E4P.

NOTE ADDED AFTER ASAP POSTING

This article was inadvertently released ASAP on 11/15/01 before final corrections were made. The word Synthase was omitted from the title. The correct version was posted 11/20/01.

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