Chorismate Mutase–Prephenate Dehydratase from *Escherichia coli*: Active Sites of a Bifunctional Enzyme†

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ABSTRACT: The relationship between the active sites of the bifunctional enzyme chorismate mutase–prephenate dehydratase has been examined. Steady-state kinetic investigations of the reactions with chorismate or prephenate as substrate and studies of the overall conversion of chorismate to phenylpyruvate indicate that there are two distinct active sites. One site is responsible for the mutase activity and the other for the dehydratase activity. Studies of the overall reaction using radioactive chorismate show that prephenate, which is formed from chorismate, dissociates from the mutase site and equilibrates with the bulk medium before combining at the dehydratase site. No evidence was obtained for direct channeling of prephenate from one site to the other, or for any strong interaction between the sites.

The biosynthesis of phenylalanine in microorganisms proceeds via the reactions (1) chorismate → (2) prephenate → (3) phenylpyruvate → phenylalanine. Reaction 1 is catalysed by chorismate mutase (EC 5.4.99.5) and reaction 2 by prephenate dehydratase (EC 4.2.1.51). In *Escherichia coli, Aerobacter aerogenes, Salmonella typhimurium,* and *Alcaligenes eutrophus* both activities are associated with a single, bifunctional protein (Cotton & Gibson, 1965; Davidson et al., 1972; Schmit & Zalkin, 1969; Friedrich et al., 1976). However, this arrangement is not universal as the two enzymes are distinct proteins in *Claviceps papsali* (Lingens et al., 1967), *Streptomyces aureofaciens* (Görisch & Lingens, 1973), *Bacillus subtilis* (Lorence & Nester, 1967; Jensen, 1969), and *Pseudomonas aeruginosa* (Waltho, 1973).

Bifunctional enzymes can be divided into two classes according to whether they catalyze sequential or nonsequential reactions along a metabolic pathway (cf. Kirschner & Bisswanger, 1976). The former class, of which chorismate mutase–prephenate dehydratase is an example, can be further subdivided into three categories on the basis of the spatial relationship between their catalytic activities. The two reactions could occur at a single active site or at two active sites which may be either separated or juxtaposed so as to permit direct transfer (channeling) of the intermediate metabolite from one site to the other. Isocitrate dehydrogenase is an example of an enzyme which utilizes a single active site to catalyze two separate reactions: the oxidation of isocitrate to oxalosuccinate and the decarboxylation of oxalosuccinate to α-ketoglutarate (Siebert et al., 1957). Many enzymes whose reaction pathways include an enzyme-bound intermediate should be considered as bifunctional, although they are not generally recognized as such. Phosphoribosylanthranilate isomerase–indoleglycerol phosphate synthase from *E. coli* (Creighton, 1970) and *Neurospora crassa* (Gaertner et al., 1970) as well as tryptophan synthase from *N. crassa* (De Moss, 1962; Matchett, 1974) appear to possess two separate active sites, and there is evidence for channeling of the intermediate between the sites for each of the *Neurospora* enzymes.

The results of previous studies on chorismate mutase–prephenate dehydratase have suggested that reactions 1 and 2 occur at separate active sites. Thus, Schmit et al. (1970), Dayan & Sprinson (1971), and Baldwin (1974) have obtained mutant enzymes which exhibit only mutase or dehydratase activity. Further, it has been demonstrated that the two activities are subject to differential inhibition (Schmit et al., 1970; Baldwin, 1974) and selective chemical inactivation (Schmit et al., 1970; Gething, 1973). However, these results do not exclude the possibility that different amino acid residues in a single active site may be responsible for each of the reactions. This report describes kinetic experiments which were undertaken to determine whether the catalytic activities of chorismate mutase–prephenate dehydratase from *E. coli* are located at one or two active sites. The data indicate that there are two separate active sites which are arranged in such a way as to preclude channeling of prephenate from one site to the other and that there is little, if any, interaction between the sites.

Experimental Section

Materials. Chorismate mutase–prephenate dehydratase was isolated from JP492, a mutant of *E. coli* K-12 (F^-, aroF363, pheO352) by the method of Gething et al. (1976). Chorismic acid was isolated from the culture medium of *A. aerogenes* strain 62-1 (Gibson, 1968). Radioactive chorismic acid was prepared in the same way, except that medium B was supplemented with 500 μCi per L of [U-^{14}C]glucose. Sodium prephenate was prepared from chorismic acid by the method of Dudziński & Morrison (1976). Other chemicals were high purity preparations from commercial sources.

Enzyme Assays. Chorismate mutase was assayed by following the decrease in absorbance at 273 nm (Koch et al., 1972). Prephenate dehydratase was assayed using a stopped time assay which involved the estimation of phenylpyruvate as described by Gibson (1964). The time periods chosen were sufficiently small that steady-state velocities were determined. The assays were performed at 37 °C in a medium containing 1.0 mM EDTA, 1.0 mM dithiothreitol, 100 μg per mL of bovine serum albumin, 0.054 M *N*-tris(hydroxymethyl)-methyl-2-ethanesulfonic acid, and 0.1 M imidazole (pH 7.5).

Estimation of Prephenate and Phenylpyruvate. The overall conversion of chorismate to prephenate and phenylpyruvate was determined at 37 °C in the same medium as that used for enzyme assays. The reaction was started by the addition of enzyme, and, after appropriate intervals, samples (0.75 mL) were withdrawn and the reaction was stopped by the addition

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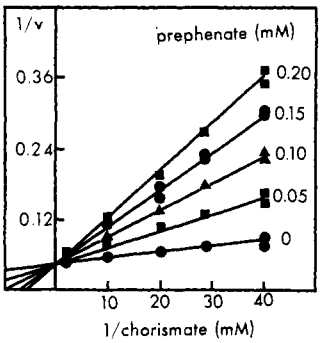

FIGURE 1: Linear competitive inhibition of chorismate mutase by prephenate. Velocities are expressed as units per mg of protein. Many of the duplicates and most of the data obtained at the highest concentration of chorismate have been omitted for the sake of clarity.

of an equal volume of 1-propanol/water (2/1, by volume). Aliquots (0.25 mL) of the diluted samples were mixed firstly with 3.0 mL of 0.9 M NaOH and then with 0.25 mL of 1 M HCl after which phenylpyruvate was estimated from the absorbance at 320 nm. The molar extinction coefficient of phenylpyruvate was taken to be 17 500. The sum of prephenate plus phenylpyruvate was determined in a similar manner, except that the HCl was added first and the solution incubated at 20–25 °C for 10 min before the addition of NaOH. In experiments which did not require the subsequent separation of chorismate, prephenate, and phenylpyruvate (see below), the addition of propanol/water was omitted, and the reaction stopped by the addition of HCl or NaOH, as appropriate.

Separation and Counting of Chorismate, Prephenate, and Phenylpyruvate. For the studies of the conversion of [U-^{14}C]chorismate to phenylpyruvate in the presence of unlabeled prephenate, the conditions and procedures for determining the concentrations of reactants were as described above. The incorporation of radioactivity from chorismate into phenylpyruvate was determined after separation of phenylpyruvate by high-voltage paper electrophoresis at pH 8.9. Aliquots (0.3 mL) of the propanol-treated samples were applied to Whatman 3 MM paper and subjected to electrophoresis in ammonium carbonate (2%, w/v) for 1 h at 25 V per cm. The mobilities of phenylpyruvate and chorismate, relative to prephenate, were found to be 0.75 and 0.95, respectively, under these conditions. Regions corresponding to phenylpyruvate, and to chorismate plus prephenate, were cut out and the radioactivity determined by scintillation counting in the toluene–Triton scintillant of Benson (1966).

Data Analysis and Simulation. Computations were performed using programs written in Focal and run on a PDP-8/I, or written in Fortran and run on a Univac 1100/42 computer. Programs for the analysis of steady-state velocity data were based on those of Cleland (1967). The analysis of progress curve data was performed using the PROCURA program (Duggleby & Morrison, 1977). Programs for the numerical integration of sets of differential equations used a second-order Runge-Kutta method (McCracken & Dorn, 1964) and were written in Focal in this laboratory. The simulation of various models for the possible channeling of prephenate between two catalytic sites was performed using programs written in Focal. The computations involved are set out in detail in the Appendix.

Results

Determination of the Kinetic Constants for the Mutase and Dehydratase Reactions. As a prelude to the study of the relationship between the active sites of chorismate mutase–prephenate dehydratase, the kinetic constants for the individual reactions were determined. Since each activity has a single substrate, it was necessary to determine only a single Michaelis constant for each reaction, and the inhibition constants for the other reactants. Steady-state velocity studies of chorismate mutase showed that prephenate acts as a linear competitive inhibitor (Figure 1). It should be noted that, because the Michaelis constant for prephenate as a substrate for the dehydratase reaction is so much greater than the inhibition constant for prephenate as a product inhibitor of the mutase reaction (Table I), there were negligible changes in the concentration of prephenate due to its conversion to phenylpyruvate. The mutase was not inhibited by phenylpyruvate at concentrations up to 6 mM. Under the conditions employed, an inhibition constant of less than 30 mM would have resulted in noticeable inhibition.

TABLE I: Kinetic Constants of Chorismate Mutase and Prephenate Dehydratase.[a]

Kinetic constant	Reaction	
	Mutase	Dehydratase
V (U/mg)	22.1 ± 0.2	18.0 ± 0.2
K(chorismate) (mM)	0.024 ± 0.001	26.0 ± 10.4[b]
K(prephenate) (mM)	0.031 ± 0.002	0.47 ± 0.01
K(phenylpyruvate) (mM)	>30	4.6 ± 0.6

[a] Kinetic constants are reported as the value ± standard error. Steady-state velocity studies were used to obtain all the kinetic constants for the mutase reaction, as well as the value for chorismate as an inhibitor of the dehydratase reaction. The other constants were determined by progress curve analysis (Duggleby & Morrison, 1977). [b] In order to prevent significant changes in the concentration of prephenate due to conversion of chorismate by the mutase, prephenate was used at a relatively high concentration. Consequently, the inhibition by chorismate was slight and only an approximate value could be obtained for the inhibition constant. For the calculations, the inhibition was assumed to be competitive.

Prephenate dehydratase is inhibited by chorismate, but only an approximate value of 26 mM (Table I) could be determined for its inhibition constant. This was due to the fact that prephenate had to be used at a concentration which was high relative to its Michaelis constant, in order to ensure that any prephenate arising from chorismate would have only a negligible effect in altering the substrate concentration. However, under these experimental conditions, the inhibition by chorismate is weak. The remaining kinetic parameters of the dehydratase have been previously determined in this laboratory by progress curve analysis (Duggleby & Morrison, 1977) and the values are listed in Table I.

Kinetics of the Overall Reaction. The kinetics of the overall reaction were studied by incubating the enzyme with chorismate and monitoring the appearance of prephenate and phenylpyruvate as a function of time. The results (Figure 2A) show that a considerable proportion of the chorismate is released as prephenate. It should be noted that the maximum rate of phenylpyruvate formation occurred after 10–12 min at which time the prephenate concentration was maximal. The lag in the formation of phenylpyruvate from chorismate is shown in more detail in an experiment using a lower concentration of enzyme (Figure 2B).

Since the kinetic constants of the individual reactions are known, it was possible to simulate the overall reaction by numerical integration and for the purposes of this simulation, it was assumed that the enzyme has two, noninteracting active sites; that is, the reactions were considered to be equivalent to those which occur in coupled enzyme systems, except that the

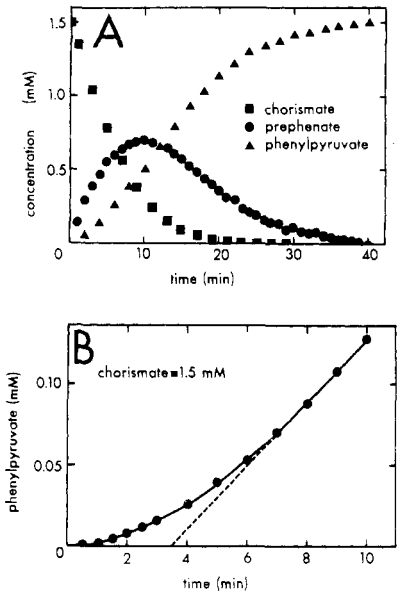

FIGURE 2: Kinetics of the overall reaction catalyzed by chorismate mutase-prephenate dehydratase. The enzyme was incubated with 1.5 mM chorismate, and the concentrations of the various components were determined in samples withdrawn at intervals. (A) The overall reaction. (B) The lag period.

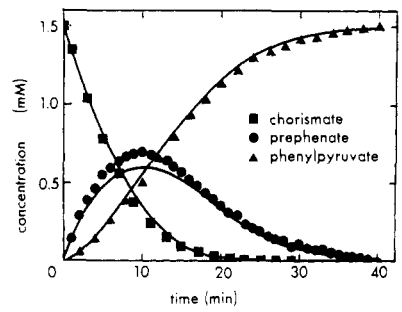

FIGURE 3: Computer simulation of the overall reaction. The data are the same as for Figure 2A. The lines are a numerical integration of the differential rate equations defined by the kinetic parameters in Table I.

activity of the second enzyme in the sequence was not far greater than that of the first. When the theoretical curves so obtained are superimposed on the data of Figure 2A, it is apparent (Figure 3) that there is good general agreement between the predicted behavior and the experimental results. The most prominent deviation occurs in the region where the concentration of prephenate reaches its highest levels.

Conversion of [^{14}C]Chorismate to Phenylpyruvate in the Presence of Unlabeled Prephenate. To investigate further the interrelations between the two catalytic activities, the effect of unlabeled prephenate on the conversion of [^{14}C]chorismate to phenylpyruvate was determined. From a reaction mixture containing enzyme, radioactive chorismate and unlabeled prephenate samples were withdrawn at intervals for determination of the chemical concentrations of the various components and, after separation by electrophoresis, the distribution of radioactivity (see Experimental Section). The chemical concentrations of the components as a function of time are illustrated in Figure 4A, together with the fitted polynomials that were used to smooth the data (see Appendix). Figure 4B shows the percentage of the total radioactivity of the added chorismate which appears in phenylpyruvate during the course of the reaction, as well as simulated curves for the expected distribution of radioactivity assuming either no channeling or

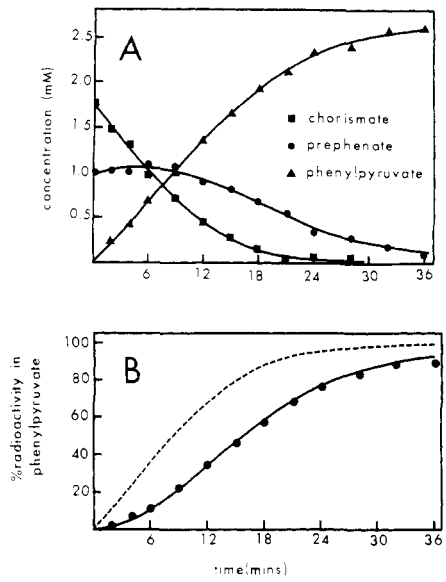

FIGURE 4: The conversion of chorismate to phenylpyruvate in the presence of added prephenate. The enzyme was incubated with 1.76 mM [U-^{14}C]chorismate (12 000 cpm per μmol) and 0.98 mM unlabeled prephenate. Samples were withdrawn at intervals and treated as described in Experimental Section. (A) Chemical concentrations of the reactants. The lines represent a least-squares fit to the data by a pair of fourth-order polynomials in time. (B) Appearance of radioactivity in phenylpyruvate. The points represent the experimental data, expressed as the percent of the total radioactivity associated with phenylpyruvate. The lines are computer simulations of the expected results, assuming either no channeling (solid line), or maximum channeling (broken line). Details of the computations are set out in the Appendix.

maximum channeling. The data lie very close to the line for no channeling and, as the simulated curves are well separated, there can be little doubt which model the data favor. Independent experiments, in which prephenate was converted to phenylpyruvate by treatment with acid prior to electrophoretic fractionation, showed that the specific radioactivity of chorismate remains constant.

Discussion

Various models may be proposed for a bifunctional enzyme which catalyzes two sequential reactions and the data obtained for chorismate mutase-prephenate dehydratase will be considered in relation to the predictions of these models. If a single site is responsible for catalyzing both reactions, prephenate may not be released, may be partially released, or may be subject to obligatory release from the active site. The first of these possibilities cannot apply since studies on the overall conversion of chorismate to phenylpyruvate show that considerable amounts of prephenate are released into solution (Figure 2A) and, moreover, phenylpyruvate production proceeds with a distinct lag (Figure 2B).

The one site model involving partial release of prephenate is set out in detail in Figure 5. Simulation studies (Figure 6) showed that this model can account for the rise and subsequent fall in the concentration of prephenate, as well as the lag in the formation of phenylpyruvate. Thus the model is in qualitative agreement with the data of Figure 2. It is of special interest that this type of behavior, which is often regarded as being diagnostic of two separate sites, can be exhibited by a one site model. The steady-state rate equation for the model shown in Figure 5 was derived by the methods of both Fromm (1970) and King & Altman (1956). Allowance was made for the irreversibility of the mutase and dehydratase reactions by assuming that $k_2k_4 = k_6k_8 = 0$. From the rate equation it was

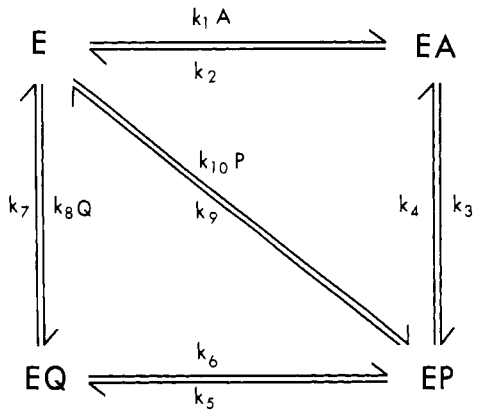

FIGURE 5: Diagrammatic representation of the model for a bifunctional enzyme with a single active site which catalyzes the conversion of A to P as well as P to Q and which may release the intermediate, P.

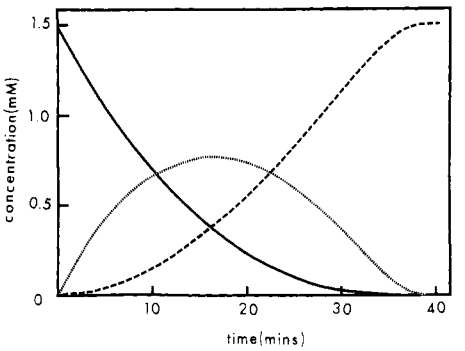

FIGURE 6: Numerical integration of the model depicted in Figure 5. The system was simulated using the following constants: K_a, 0.03 mM; K_p, 0.05 mM; and K_q, 5 mM. The initial concentration of A was taken to be 1.5 mM, and the relative maximum velocity of the first activity to that of the second was set at 1.25:1. The total concentration of enzyme was chosen to give a convenient time scale. The lines represent the concentrations of A (solid line), P (dotted line), and Q (broken line).

apparent that the kinetic parameters for prephenate as a product inhibitor of the mutase reaction and as a substrate for the dehydratase reaction are identical and equal to the expression

$$\frac{(k_2 + k_3)(k_5k_7 + k_6k_9 + k_7k_9)}{k_{10}[(k_2 + k_3 + k_4)(k_6 + k_7) + k_5(k_2 + k_3)]}$$

Further, it was evident that the kinetic parameters for phenylpyruvate as a product inhibitor of the dehydratase reaction and as a dead-end inhibitor of the mutase reaction are identical and given by the expression

$$\frac{k_5k_7 + k_6k_9 + k_7k_9}{k_8(k_5 + k_9)}$$

With the more restrictive assumption that $k_6 = 0$, this expression becomes k_7/k_8, a true dissociation constant. The predicted equalities are not supported by the data (Table I). The Michaelis constant for prephenate is 15 times greater than its product inhibition constant and phenylpyruvate inhibits the dehydratase reaction more strongly than the mutase reaction. Thus, a one site model with partial release of prephenate may be eliminated on quantitative grounds.

If a single site model involving obligatory release of prephenate is to be entertained, it is necessary to consider reasons for the obligatory release. Three possible reasons are: (a) the enzyme must undergo an isomerization from a "mutase form" to a "dehydratase form" and this isomerization cannot take place while the active site is occupied by prephenate; (b) prephenate formed by the action of the mutase is not in the correct orientation for the dehydratase and must be released and rebound in the proper orientation; (c) prephenate formed by the mutase is in the wrong isomeric form and must be released, isomerized, and be rebound before it can act as a substrate for the dehydratase. The steady-state rate equations for these various models were derived and it was noted that each equation predicted the equality of the Michaelis constant and product inhibition constant for prephenate, as well as the equality of the product and dead-end inhibition constants for phenylpyruvate. Since these equalities are not observed (Table I), single site models involving obligatory release of prephenate are eliminated.

In the foregoing discussion, it has been shown that no single site model will account for the observed kinetic properties of chorismate mutase–prephenate dehydratase. Thus, two active sites must be involved in the conversion of chorismate to phenylpyruvate via prephenate, and several two site models have been considered. These models place no constraints on the relative magnitudes of the various kinetic constants, so the data in Table I cannot distinguish between the various possibilities and other properties must be examined in order to select the correct model. The two active sites of a bifunctional enzyme could be independent or subject to protein-mediated interaction. In addition, the two sites could be juxtaposed so as to permit channeling of the intermediate between the sites. Channeling for a two site bifunctional enzyme is envisaged as the direct transfer of an intermediate from one site to another where it undergoes further conversion. In the reaction A → P → Q, complete channeling would involve the direct conversion of A to Q without any release of P from the site at which it is formed. On the other hand, channeling could occur together with the partial release of P from one or both of the catalytic sites. In this case, the operational definition of channeling would be that P does not exchange completely with any P present in the medium. The degree of channeling must depend on the relative magnitude of the rates for the partitioning of P between transfer and release at the first site, and between conversion and release at the second site. The whole concept of channeling is developed in detail in the Appendix. If channeling does occur, it would be expected that in the overall reaction, labeled prephenate derived from [^{14}C]chorismate would not equilibrate freely with added unlabeled prephenate. The results (Figure 4) demonstrate clearly that little, if any, of the prephenate is channeled from the mutase to the dehydratase site and hence the two sites are distinct and separate. The possibility that the two sites might interact through protein-mediated effects was investigated by simulating the overall conversion of chorismate to phenylpyruvate by numerical integration using the kinetic parameters determined from studies of the individual reactions. While there is good agreement between the predicted curves and the experimental data (Figure 3), there were some discrepancies which could not be removed by variation of the kinetic parameters by ±50% of their value. The discrepancies may be indicative of some interaction between the sites. In a complementary analysis an attempt was made to perform a nonlinear least-squares fit to the data of Figure 2A, using the CRICF program (Chandler et al., 1972). It was hoped to compare the kinetic constants evaluated in this way with those reported in Table I. Unfortunately, as it was not possible to obtain convergence in the program, such a comparison could not be made.

The results of the present investigation, together with those of earlier studies on the differential inhibition and inactivation of the two activities, as well as the isolation of monofunctional

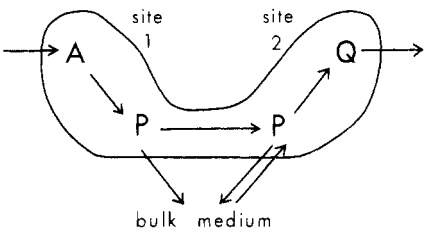

FIGURE 7: Diagrammatic representation of a two site bifunctional enzyme which permits channeling of the intermediate, P.

mutant enzymes (Schmit et al., 1970; Gething, 1973; Baldwin, 1974), leave little doubt that chorismate mutase–prephenate dehydratase possesses two separate and distinct active sites. Further, the kinetic data indicate that any interaction between the sites is small. It is interesting to note that, if the discrepancies between the data and the theoretical curves in Figure 3 are indicative of intersite interactions, then these interactions actually promote the accumulation of prephenate. Less detailed investigations have been made of the kinetic properties of chorismate mutase–prephenate dehydratase from *S. typhimurium* and from *A. eutrophus,* but studies of the overall conversion of chorismate to phenylpyruvate (Schmit & Zalkin, 1969; Friedrich et al., 1976) have shown that with both enzymes there is release of prephenate into solution as well as a lag in phenylpyruvate production.

The possession by organisms of bifunctional enzymes or bienzyme complexes which do not permit the release of an intermediate product may offer evolutionary advantages. The amount of the second enzyme required to maintain a particular metabolic flux could well be less than with a coupled system composed of noninteracting enzymes. More important perhaps, the intermediate is not subject to degradation or diversion into another pathway. The bienzyme complex carbamoyl phosphate synthase–aspartate transcarbamoylase (Lue & Kaplan, 1970) and the bifunctional tryptophan synthase from *N. crassa* (Gaertner et al., 1970) catalyze sequential metabolic reactions with little or no release of the intermediate product. By contrast, chorismate mutase–prephenate dehydratase, like phosphoribosylanthranilate isomerase–indoleglycerol phosphate synthase (Creighton, 1970) behaves in a manner which is virtually identical to that expected for an equimolar mixture of two monofunctional enzymes. Thus the evolutionary advantage of the bifunctional nature of these proteins is problematical. In this connection, it should be noted that Yourno (1972) has isolated mutants of *S. typhimurium* in which the His C and His D genes have fused to give a bifunctional imidazoleacetol phosphate aminotransferase–histidinol dehydrogenase. Rechler & Bruni (1971) have shown that the kinetic properties of this bifunctional enzyme differ little from those of the corresponding monofunctional enzymes. Thus, it may be that the accidental fusion of chorismate mutase and prephenate dehydratase is retained in some organisms simply because it is not detrimental. However, it is possible that the advantage of the bifunctional nature of chorismate mutase–prephenate dehydratase is related to its regulatory properties. Each activity is strongly inhibited by phenylalanine (Dopheide et al., 1972; Schmit & Zalkin, 1969) which appears to bind at a third site on the enzyme (Schmit et al., 1970). Thus, if any significance can be attached to the occurrence of both activities on the same protein molecule, it is perhaps that this arrangement permits coordinate inhibition of both activities by phenylalanine. It is of interest that the monofunctional dehydratase from *B. subtilis* (Rebello & Jensen, 1970) is regulated by phenylalanine, whereas the monofunctional mutases from *Streptomyces aureofaciens* (Görisch & Lingens, 19' 3) and *B. subtilis* (Lorence & Nester, 1967) are not affected by this amino acid. However, some monofunctional mutases do show regulatory properties. Those from *Euglena gracilis* (Weber & Bock, 1970) and *Claviceps papsali* (Sprössler & Lingens, 1970) are inhibited not only by phenylalanine, but also by tyrosine.

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Appendix

The Channeling Model. The notion of channeling of an intermediate between two enzymes or between two active sites of a bifunctional enzyme was considered by Davis (1967), but over the past decade there has been little further development of the concept. In this discussion the general concept of channeling will be considered and attention directed to some of the more quantitative aspects of the topic. While comments will be confined to the case where two different active sites reside on a single polypeptide chain, they could equally well apply to a system involving two or more associated enzymes.

A diagrammatic representation of the general channeling concept for a bifunctional enzyme is illustrated in Figure 7. The essential features of the concept are that: (a) there are two active sites. At site 1, A is converted to P and at site 2, P is converted to Q; (b) after formation of P, the intermediate is partitioned between release into the bulk medium and direct transfer (channeling) to site 2; (c) after combining at site 2, P can be further partitioned between release into the bulk medium and conversion to Q which then dissociates from the enzyme.

The degree of channeling observed will depend on the relative magnitude of the rates of the various steps. In certain limiting cases there are no conceptual difficulties about defining either complete channeling or no channeling. Thus, if the dissociation of P from site 1 is far more rapid than its transfer to site 2, so that P mixes completely with the bulk medium, then there can be no channeling. On the other hand, if transfer occurs without the release of P, then there is complete channeling, although the degree of channeling observed will depend on the rates of conversion and release of P at site 2. For example, if there is complete channeling but the rate of conversion of P to Q is much higher than those for transfer and release, then the formation of Q from A will be supplemented by the production of Q from any P present in the bulk medium. Alternatively, if the conversion of P to Q is slow, compared with the rate of transfer, P will dissociate from site 2 and complete channeling will not be observed. Indeed, this situation cannot be distinguished from that involving the partial release of P from site 1.

Definitions of Channeling. The aforementioned considerations draw attention to the difficulties associated with the measurement of the true degree of channeling. In terms of the model described in Figure 7, channeling is the transfer of the intermediate P from site 1 to site 2. Therefore, the mechanistic definition of channeling would relate to the degree of partitioning of P at site 1 between intersite transfer and dissociation from the enzyme. Since channeling must be detected by a measurement related to the release of P into the bulk medium and as P can be released from site 2 as well as site 1, the mea-

sured amount of channeling could be considerably lower than that expected on the basis of the partitioning of P at site 1. The measured value must, therefore, be regarded as the operational degree of channeling which will always be an apparent value unless the conversion of P to Q is very rapid compared with transfer and release of P at site 2. At present, it is difficult to envisage an experimental method which would permit measurement of mechanistic channeling.

Detection of Channeling. The experimental approaches to determination of the degree of channeling involve either the trapping of the intermediate or its exchange with intermediate added to reaction mixtures. The intermediate may be trapped chemically by the addition of a noninhibitory compound with which it specifically and rapidly reacts to give rise to an unreactive complex, or enzymically by use of high concentrations of a coupling enzyme which diverts the intermediate into a different product. The operational channeling would be determined directly from the proportion of substrate yielding product relative to the total conversion of substrate. The exchange procedure involves carrying out the reaction with radioactive substrate in the presence of unlabeled intermediate (or vice versa). Operational channeling would now be determined by following the time course of the reaction with respect to changes in the chemical concentrations of the reactants, as well as the distribution of radioactivity, and simulating the overall reaction for various extents of channeling as described below. An alternative procedure would be to have present in a reaction mixture highly labeled intermediate with unlabeled substrate and to determine the specific radioactivity of the product formed in the early stages of the reaction where the operational channeling would be inversely related to the amount of radioactivity appearing in the final product.

It is important to note that the results from studies with a competing coupling enzyme could differ from those obtained by using either a chemical trapping agent or radioactive reactants. Such a difference would arise if the small molecules were able to react or exchange with the intermediate through their abilities to penetrate a region in the vicinity of the active sites to which the very much larger enzyme molecule did not have access.

In this report, the appearance of radioactivity in the final product was monitored in a system containing radioactive initial substrate and unlabeled intermediate. The extent of channeling was determined by simulating the system for various extents of channeling, and selecting the model which most closely approximated the data.

Simulation of the Channeling Model. To simulate the model described above so that the extent of channeling could be determined, it is necessary to know the rates of reaction at each active site at any particular moment in time. While such information could be obtained from a theoretically based and quantitatively accurate description of the kinetic properties of the system, such a description may not easily be acquired. Indeed, it may be necessary to know the extent of partitioning at each site in order to develop such a model. In order to avoid this problem, the simulation was achieved in two stages. First, the chemical data on the progress of the reaction were smoothed by fitting to an empirical equation. For the particular experiment described in Figure 4, the data for chorismate and those for phenylpyruvate were each fitted to a fourth-order polynomial in time. As the sum of the concentrations of all three components must be a constant, the coefficients of the smoothing polynomial for the prephenate data were obtained by difference. Equations involving sums of exponentials were tried, but these did not give a satisfactory fit to the data. With an empirical description of the chemical data at hand, the appearance of radioactivity in the final product was simulated in a stepwise fashion, with a step size which was sufficiently small so as to accurately represent a continuous process. In each step, the decrease in concentration, and therefore the disappearance of radioactivity from the initial substrate were calculated from the smoothing equations, and this was distributed between the bulk medium and direct transfer to the dehydratase site, according to the partitioning at site 1 being simulated. Then the amount of intermediate at the second site was adjusted by either further release or supplementation from the bulk medium, depending on whether it was greater or less than, the increase in phenylpyruvate concentration calculated from the smoothing equations. The amount of radioactivity at the second site and in the bulk medium was adjusted in a comparable fashion. Finally, the prephenate at site 2 was moved into the phenylpyruvate pool, and the radioactivity in phenylpyruvate calculated, taking note of the fact that there is a 10% loss of radioactivity due to release of carbon dioxide. In this way, the appearance of radioactivity in the final product may be calculated as a function of time for any chosen amount of mechanistic channeling. The computer program for this simulation was written in Focal in this laboratory.

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Structural Similarities and Differences among Metal Ion Complexes of Phosphoglucomutase by Solvent Perturbation and Ultraviolet Difference Spectroscopy†

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ABSTRACT: Although the binding of bivalent metal-ion activators to phosphoglucomutase produces substantial changes in the near ultraviolet spectrum of the enzyme, the extent to which aromatic residues are exposed to the aqueous environment, as assessed by means of solvent perturbation spectroscopy (using D_2O), does not appear to be significantly altered by the binding process. Other ways in which the spectral effects induced by activation might arise are considered by making comparisons with those changes induced by various nonactivating monovalent and bivalent cations. The observed differences are most easily interpreted in terms of an electrostatic perturbation of (at least) two different tryptophan residues. This interpretation is supported by using cationic vs. neutral (zwitterionic) tryptophan in various solvent systems to generate difference spectra that are similar either to the observed metal-ion induced spectral differences or to the differences in the spectral changes produced by various pairs of metal ions. Although a rationale for the striking similarity in the spectral changes produced by Mg^{2+} and by Li^+ (which elicits less than 2×10^{-8} of the enzymic activity induced by Mg^{2+}) cannot be ascribed to a simple electrostatic effect, alone, the involvement of an additional, negatively charged group in the binding of Mg^{2+} (but not Li^+) could reduce the effective charge of bound Mg^{2+} to a value close to that of bound Li^+.

An earlier paper (Ray et al., 1978) describes the unusually tight binding of Li^+ to phosphoglucomutase in the presence of its substrates (glucose phosphates). In fact, bound substrates increase the binding of Li^+ by some 900-fold, although the binding of Mg^{2+}, the normal activator, scarcely is altered under analogous conditions. These observations indicate that there are important structural differences between the E_P·Li and E_P·Mg complexes,[1] a conclusion that is supported by the more than 5×10^7-fold difference in their catalytic activities (in the presence of bound substrates). Hence, it was surprising to find that the spectral change induced by the binding of Li^+ to the enzyme is quite similar to that induced by Mg^{2+}, although other monovalent cations produce spectral changes that are quite different in appearance. The similarity in the spectral change induced by Mg^{2+} and Li^+ is not consistent with the earlier suggestion (Peck & Ray, 1969) that such spectral differences are produced by the conformational changes upon which the activation process depends—changes which were thought to alter the exposure of aromatic residues to the aqueous environment. The present paper suggests, instead, that metal-ion induced spectral changes in this system arise primarily from electrostatic effects, not from changes in exposure to the solvent, and that a charge-compensating effect may accompany the binding of metal ion activators such as Mg^{2+}.

Experimental Section

Phosphoglucomutase was isolated, assayed, freed of contaminating bivalent metal ions, and stored according to previously described procedures (cf., Ray et al., 1978). UltraPure sodium, potassium, and cesium chlorides, and UltraPure magnesium nitrate were obtained from Ventron; lithium chloride was crystallized once from 20 mM EDTA and twice from water and was dried under vacuum over P_2O_5 before use. Reagent grade tetramethylammonium chloride was used,

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[1] Abbreviations are: E_P and E_D, the phospho and dephospho forms of phosphoglucomutase; E_P·M, the metal-ion complex in which M^{2+} is bound at the metal-ion activating site.

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