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Influence of an aggregated multienzyme system on transient time: Kinetic evidence for compartmentation by an aromatic-amino-acid-synthesizing complex of *Neurospora crassa*

(analog computer/channeling *in vitro*/multienzyme complex)

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ABSTRACT **The aromatic complex of *Neurospora crassa* is an aggregated multienzyme system which catalyzes five consecutive reactions in the central pathway leading to the biosynthesis of the aromatic amino acids. In an attempt to understand the physiological importance of this complex in particular, as well as the importance of cellular organization of enzyme systems in general, we have isolated the complex and have begun to characterize its catalytic properties. Optimum conditions for the assay of the overall 5-step reaction catalyzed by the partially purified complex have been determined. An analog computer was programmed to represent an unaggregated system of five enzymes with rate constants identical to those found for the constituent enzymes of the complex. By direct comparison, it was shown that the lags (transient times) obtained for the overall reaction were 10–15 times longer for the hypothetical unaggregated system than for the complex. We conclude from these data that the aggregated multienzyme system compartmentalizes intermediate substrates during the course of the overall reaction. We suggest that, in addition to "channeling" intermediates of competing pathways, reduction of the transient time may be an important consequence of the containment of intermediates within a physically associated enzyme sequence. The fact that the aromatic complex exhibits a second catalytic property unique to aggregated enzyme systems, "coordinate activation" [Welch, G. R. & Gaertner, F. H. (1975) *Arch. Biochem. Biophys.*, in press] indicates that the physical association of these enzymes may have more than one physiological function.**

A multienzyme system may be defined simply as a group of two or more consecutive enzymes of a metabolic sequence (1). It has been suggested (2–6) that the primary physiological role of aggregated multienzyme systems (i.e., multienzyme complexes) may lie in their ability to facilitate catalysis over the corresponding unaggregated systems, and that this facilitation might involve such features as (*a*) coordinated activation or inhibition of constituent enzymes and (*b*) compartmentation of intermediate substrates. As a consequence of either activation or compartmentation, one would expect (with a complex, as compared with an unaggregated system) to observe a reduction of the transient time (1, 7, 8) for the overall multienzyme system, as well as a depression of the levels of free intermediate substrates.

In another report (9), we have shown that the five-enzyme aromatic complex of *Neurospora crassa* exhibits the interesting property of coordinate activation by the first substrate. Here we have extended the earlier kinetic studies to examine the transient time in relation to the possibility of compartmentation of intermediate substrates by this complex.

A direct approach to the kinetic analysis of catalytic facilitation by aggregated multienzyme systems would entail a comparison of substrate accumulation and transient-time data for existing multienzyme complexes with that for the corresponding unaggregated systems (cf. 10). When the latter are not available, as is the case for the aromatic complex, one may use a suitable analytical representation.† In this case, we have used an analog computer to simulate the dynamic behavior of the aromatic complex.‡ In addition to the coordinate activation of the complex observed earlier (9), these results show a second unique kinetic property attributable to the aggregated state of multienzyme systems—reduction of the transient time.

EXPERIMENTAL METHODS

Methods for growth of *N. crassa* (strain 74A), preparation of extracts, and enzyme purification were detailed previously (9).

The five-step overall reaction, 3-deoxy-D-*arabino*heptulosonate 7-phosphate (DAHP) to 5-*enol*pyruvylshikimate 3-phosphate (ES-3-P) of the aromatic complex was measured fluorometrically in the presence of an excess of the isolated coupling enzymes, chorismate synthase and anthranilate synthase [chorismate pyruvate-lyase (amino-accepting), EC 4.1.3.2.7], as modified from Gaertner *et al.* (4, 12). The reaction mixture, except where noted, contained the following: 100 mM potassium phosphate, pH 7.0; 5.0 mM $MgSO_4$; 0.5 mM NAD; 1.0 mM DAHP; 2.5 mM ATP; 3.0 mM phospho*enol*pyruvate; 5.0 mM glucose 6-phosphate; 20 mM L-glutamine; 0.1 mM NADPH; 10 μM FMN; pyruvate kinase [3.0 μmol/min (units) per ml]; glucose-6-phosphate dehydrogenase (5 units/ml); and aromatic-complex sample in a total volume of 0.1 ml. Anthranilate synthase was purified by preparative electrophoresis (9). Chorismate synthase was either a purified preparation (13) or a DEAE-cellulose fraction void of aromatic complex (9). Upon addition of enzyme sample the increase in fluorescence due to anthranilate was

Abbreviations: DAHP, 3-deoxy-D-*arabino*heptulosonate 7-phosphate; ES-3-P, 5-*enol*pyruvylshikimate 3-phosphate.

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† The enzymes in the *N. crassa* aromatic complex appear to be dissociated in extracts from various bacteria (11). Providing favorable K_m and V_{max} relationships exist between *N. crassa* and bacterial enzyme systems, one could in principle employ the bacterial multienzyme system for comparative studies here, instead of an analytical model.

‡ The basic module employs Michaelis–Menten kinetics and is suitable for systems exhibiting simple Michaelian behavior. As seen previously (9), the enzymes of the aromatic complex are of this type.

recorded. Chorismate synthase and anthranilate synthase were assayed by methods reported elsewhere (12, 13). Protein was determined by absorbance at 280 nm. Unless otherwise stated, enzyme activities are in μmol/min (units) at 25°.

The analog computer, Electronic Associates, Inc. (EAI) 221-R, was equipped with a 100 V reference and Bi-Polar Quarter-Square Multipliers (no. 7.104) and was operated with an EAI model 1100E X-Y plotter. In the computer program (14), the aromatic-complex sequence of reactions was approximated by an irreversible set of five monomolecular reactions[§] with the correspondence S_1:DAHP, S_2:dehydroquinate, S_3:dehydroshikimate, S_4:shikimate, S_5:shikimate 3-phosphate, and P:ES-3-P. The standard Michaelis-Menten expression was used to generate the rates for each reaction in the multienzyme sequence. The V_{max} and K_m values used in the program were the same as those given in Table II of ref. 9 for the aromatic complex.

The following chemicals came from Sigma Chemical Co.: FMN, D-glucose 6-phosphate, glucose-6-phosphate dehydrogenase (crystallized preparation), and pyruvate kinase (crystalline suspension in ammonium sulfate). The following came from Calbiochem: phospho*enol*pyruvate, ATP, NAD, NADPH, and L-glutamine. DAHP was prepared enzymatically as detailed previously (9).

RESULTS

Although the aromatic complex contains five distinct enzymes, the aggregated state affords a unique situation in which to consider the aggregate as a single, physical unit of enzyme action (namely, catalyzing the overall, multistep conversion of the primary substrate DAHP to the final product ES-3-P). Of practical value, this overall activity could serve as a monitor for the structural-functional integrity of the aggregate during extraction and purification procedures. Moreover, a knowledge of the kinetic behavior exhibited by the multienzyme complex during the overall production of ES-3-P from DAHP would be of value in assessing the potential catalytic and/or physiological advantages of the aggregated state, as compared with the unaggregated enzyme system. To this end, we developed an *in vitro* assay for the aromatic complex, expressing the production of ES-3-P from DAHP, based on a modification of an overall assay described previously (4, 12).

A typical example of the overall reaction catalyzed by the aromatic complex which represents the appearance in time of the final product, ES-3-P, is represented in Fig. 1. The reaction exhibits a characteristic lag (transient phase) before linearity is reached.[¶] [The time-intercept of the linear portion of the curve will be referred to as the transient time (cf. 7).] Both the linear rate and transient time for the overall assay were proportional to enzyme concentration up to about 0.001 units of dehydroquinate synthase per ml of assay mixture (Table 1). Moreover, the final rate obtained in the overall assay in each case closely approached the value predicted from the independent assay of the rate-limiting first enzyme.

In a separate experiment, the activation effect of DAHP on the overall rate was studied. We found earlier (9) that

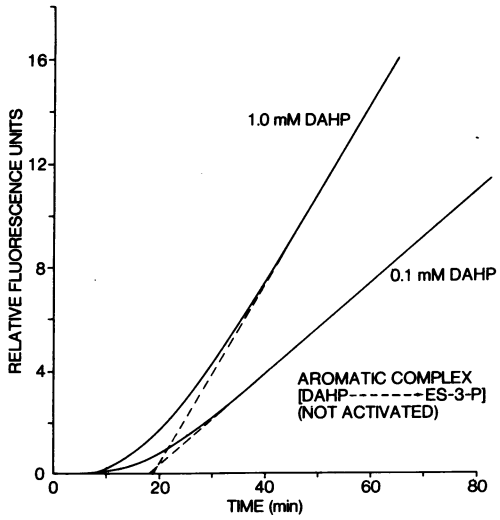

FIG. 1. Overall assay of the aromatic complex. Purified aromatic complex was assayed according to the "overall assay method" described in *Experimental Methods*, except that the DAHP concentration was 1.0 mM or 0.1 mM as indicated. (One fluorescent unit here and in Fig. 2 is equivalent to 1 nmol/ml of anthranilate.)

four of the five enzymes of the complex were activated when incubated with 5–10 mM DAHP. The concentration of DAHP for half-maximal activation was about 1.0 mM. As shown in Fig. 2, when assayed with 1.0 mM DAHP in the reaction mixture, aromatic complex previously incubated with 10 mM DAHP (minus cofactors and cosubstrates to prevent catalysis) yielded a rate of production of ES-3-P 2-fold higher than that for the nonactivated case. In addition, the transient time was reduced 2-fold. These activation effects (i.e., rate stimulation and reduction of transient time) were observed throughout the range of enzyme concentrations given in Table 1. Enzyme preincubated with 1.0 mM DAHP (and assayed at the same level) produced a final rate essentially equal to the nonactivated case even though the

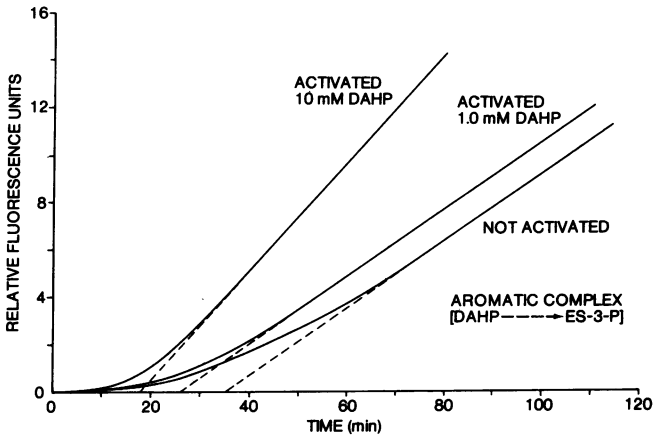

FIG. 2. Effect of DAHP activation on the overall assay of the aromatic complex. Purified aromatic complex was incubated 30 min at 0–5° in the presence of 10 mM DAHP, 1.0 mM DAHP, or 100 mM potassium phosphate, pH 7.0 (i.e., nonactivated), as indicated. For the 10 mM DAHP-activation case, the incubated sample was diluted 10-fold into the complete "overall assay" mixture, minus the DAHP component, and assayed as related in *Experimental Methods*. In the other two cases, the samples were diluted 10-fold into complete "overall assay" mixtures, containing 1.0 mM DAHP.

[§] Although some of the constituent reactions are bisubstrate in nature, the situation for monosubstrate reactions was approximated by use of saturation levels of the respective secondary substrates.

[¶] As calculated by the method of Easterby (7), the contribution to the transient time due to the coupling enzymes, chorismate synthase and anthranilate synthase, is negligible (≤1 min).

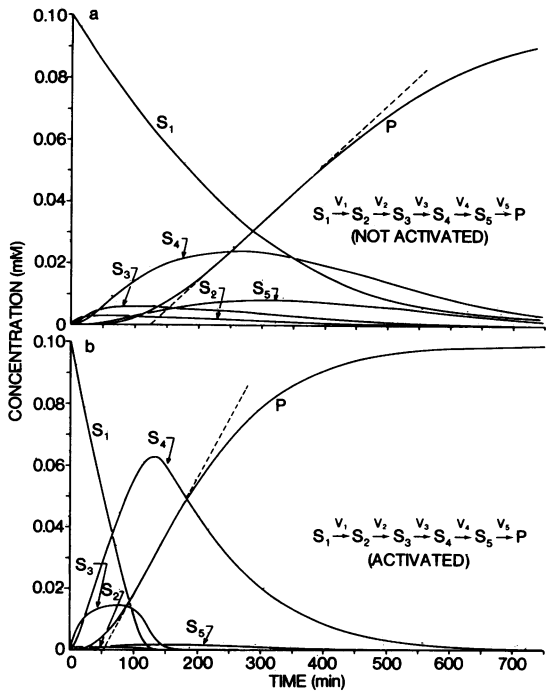

FIG. 3. Computer simulation of the kinetic behavior of a 5-step multienzyme system analogous to the aromatic-complex sequence with 0.1 mM initial condition. S_1 (0) = 0.1 mM, in the nonactivated (a) or activated (b) states. The V_{max} (V_1) of the first enzyme is set at 5×10^{-4} units/ml (nonactivated), and the remaining V_{max}s are proportional according to Table II of ref. 9. K_m values were obtained from the same table.

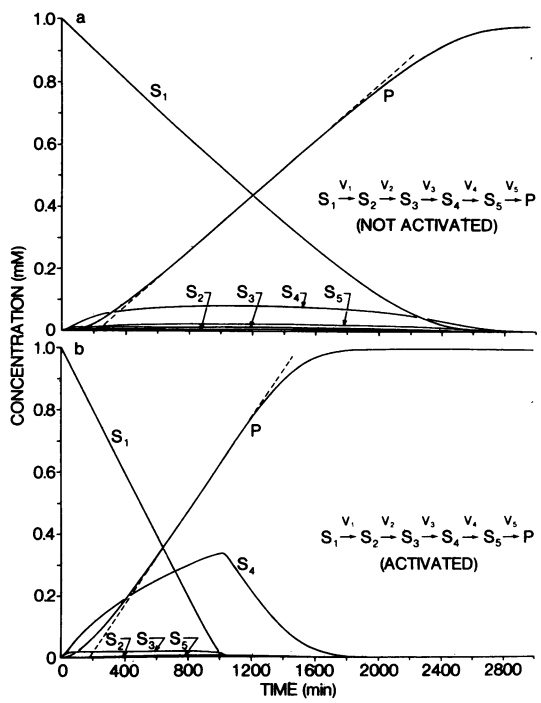

FIG. 4. Computer simulation of the kinetic behavior of a 5-step multienzyme system analogous to the aromatic-complex sequence with 1.0 mM initial condition. Details of computer simulation for the nonactivated (a) and activated (b) states are exactly as described for Fig. 3, except that S_1 (0) = 1.0 mM.

transient time was reduced about 50%. Aromatic complex preincubated with 1.0 mM DAHP and assayed at the 0.1 mM level gave a final rate identical to that preincubated with buffer alone, but the transient time was equal to that for enzyme both preincubated and assayed at 1.0 mM concentrations (results not shown). The 2-fold stimulation in the final rate of the overall reaction is consonant with the 2-fold increase in V_{max} previously observed for dehydroquinate synthase after preincubation with DAHP (9).

For computer simulation of the kinetic behavior of a five-step, monosubstrate multienzyme sequence, an enzyme concentration corresponding to 5×10^{-4} units/ml of assay mixture for the first enzyme (dehydroquinate synthase) was used. This activity represents that employed for the *in vitro* overall assays of the aromatic complex. Simulation of the kinetic behavior was performed for 1.0 mM and 0.1 mM initial concentrations of DAHP (S_1) and for activated versus nonactivated states (Figs. 3 and 4). With an initial concentration of 0.1 mM DAHP, a transient time of 125 min was predicted by the computer, whereas at the 1.0 mM level a transient time of 220 min was indicated. Upon activation, the transient time for the 0.1 mM DAHP case was reduced by 50%, while that for the 1.0 mM instance was reduced by only 25%. Of further importance from the computer studies was the prediction of accumulation of intermediate substrates, particularly shikimate, during the course of the overall reaction sequence. For example, in the activated state Figs. 3b and 4b show maximal levels of accumulated S_4 (shikimate) of about 0.06 mM and 0.35 mM for the 0.1 mM and 1.0 mM concentrations of DAHP, respectively.

In Figs. 5 and 6, data from Figs. 3 and 4 are replotted and compared with actual experimental findings for the overall aromatic complex assay (nonactivated case). These data

Table 1. Relationship of the transient time and rate to enzyme concentration for the overall aromatic-complex assay*

Dehydroquinate synthase V_{max} (units/ml × 10^4)†	1.0 mM DAHP		0.1 mM DAHP	
	d[ES-5-P]/dt (units/ml × 10^4)‡	τ § (min)	d[ES-5-P]/dt (units/ml × 10^4)‡	τ § (min)
17.0	10.0	8	10.0	7
8.5	5.0	15	5.0	13
6.0	3.6	20	1.8	20
3.0	1.7	38	0.8	38

* The aromatic complex (not activated) at various enzyme concentrations was assayed with either 0.1 mM or 1.0 mM DAHP, as indicated, according to the "overall assay" procedure described in *Experimental Methods*.

† See ref. 9 for V_{max} proportion among the aromatic-complex constituent activities. Activities are in units/ml of assay mixture.

‡ The values here represent the final linear rates achieved after the initial transient phase.

§ The transient time, τ, is defined as the time intercept of the final linear (quasisteady-state) rate (7).

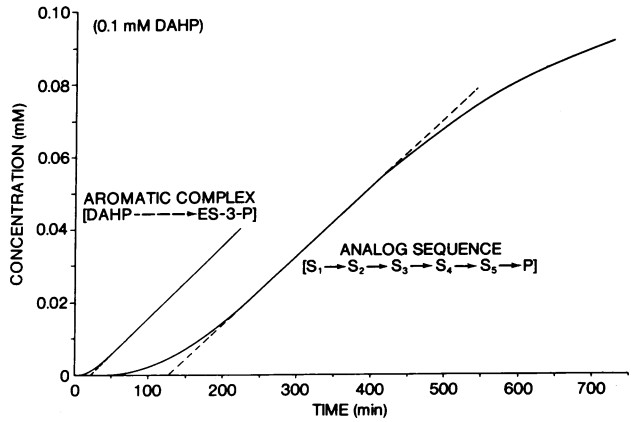

FIG. 5. Comparison of the "overall activity" of the aromatic-complex system and that predicted by computer for the hypothetical analogous multienzyme sequence with 0.1 mM initial condition. Purified aromatic complex was assayed according to the "overall assay method" detailed in *Experimental Methods*, except that the concentration of DAHP was 0.1 mM. The concentration of aromatic complex corresponded to V_{max} of 5×10^{-4} units/ml for dehydroquinate synthase. (See Table II of ref. 9, for V_{max} proportion.) For comparison, the curve of [P] versus time from Fig. 3a is replotted here.

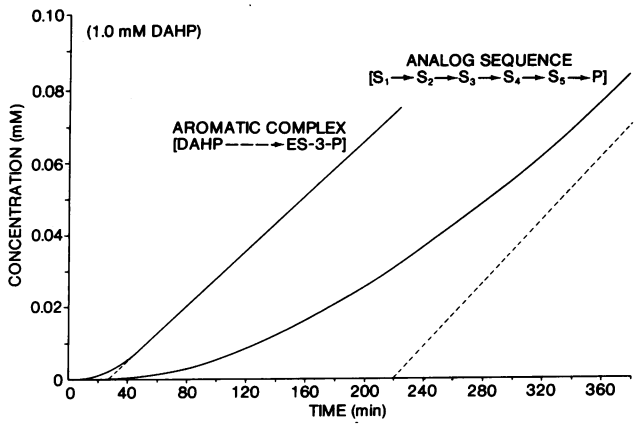

FIG. 6. Comparison of the "overall activity" of the aromatic-complex system and that predicted by computer for the hypothetical analogous multienzyme sequence with 0.1 mM initial condition. Experimental conditions are exactly as recounted for Fig. 5, except that the concentration of DAHP was 1.0 mM. The curve of [P] versus time from Fig. 4a is replotted for comparison in this case.

demonstrate a significant reduction of the transient time for the multienzyme complex, as compared with the hypothetical unaggregated system. For a 1.0 mM DAHP initial condition, the transient time was decreased about 10-fold, whereas a 5-fold reduction was observed with 0.1 mM DAHP. Although not shown in Fig. 6, the transient time for the activated aromatic complex assayed with 1.0 mM DAHP was about 12 min. Comparison of this value with that from Fig. 4b (180 min) indicates a 15-fold decrease in the transient time.

DISCUSSION

The possibility of reduction of the transient time has been predicted as an important feature of isolated multienzyme complexes (8) and membrane-associated multienzyme systems (15), when compared with the corresponding separated schemes. Moreover, extensive *in vitro* studies (16–21) with *artificially* immobilized enzymes have confirmed such predictions. Results presented here provide *in vitro* evidence of a significant reduction of the transient time for a *naturally* occurring aggregated multienzyme system (membranous or otherwise) of such complexity as the aromatic system of *N. crassa*.

For an unaggregated multienzyme system, a transient phase is expected due to the time required for intermediates to accumulate during the course of the overall reaction (7). Hence, the observed transient-time reduction by the aromatic complex is readily interpretable in terms of the containment (compartmentation) of intermediates within or on the surface of the aggregate. The fact that the transient time was equal for aromatic complex assayed with 1.0 mM and 0.1 mM DAHP (Fig. 1 and Table 1) is consistent with this idea. For example, in comparing the kinetics at the two concentrations of DAHP (Figs. 3a and 4a) the computer shows a significant difference in the profile for accumulation of intermediates and a difference in overall transient times (i.e., 125 versus 200 min). The finding of equal transient time for 0.1 mM and 1.0 mM DAHP (i.e., 20 min) is, then, a possible reflection of the same degree of compartmentation in each case. In no instance was it possible by incubation with DAHP to completely eliminate the transient phase exhibited by the *in vivo* overall assay of the aromatic complex. The explanation for the residual transient time is unknown at present. However, this period may represent the time required for certain conformational changes to occur within the enzyme aggregate, or for necessary levels of intermediate substrates to accumulate.

In the activated state, shikimate kinase (ATP:shikimate 5-phosphotransferase, EC 2.7.1.71) potentially commands by an order of magnitude the longest transient time of the system. The dramatic accumulation of shikimate predicted by the computer is a reflection of this situation. The shikimate kinase reaction in the aromatic complex clearly exhibits a significantly reduced transient time as compared with the hypothetical unaggregated scheme, and therefore a much reduced accumulation of shikimate would be predicted under actual circumstances.

For membrane-associated enzyme systems it has been shown (19) that Michaelis–Menten kinetics must be supplemented with diffusion-related terms in order to completely describe the spatio-temporal variation of substrates and products. Likewise, in aggregated enzyme systems, wherein intermediate substrates may be compartmentalized, the diffusion process may become interwoven with the stepwise catalytic process. Moreover, it is usually assumed possible to select a small enough volume containing a homogeneous solution of substrate molecules in sufficiently large number for application of Michaelis–Menten kinetics.‖ Yet, within a multienzyme complex which compartmentalizes (or "channels") intermediates, fewer than 10 substrate molecules may be involved per active site (see, for example, refs. 22 and 23). Whether these additional factors are relevant remains to be determined, but the finding of such specific active-site localizations of intermediate substrates, concomitant to elimination of interenzymic diffusion processes and associated

‖ As used here, the Michaelis–Menten kinetics employed with the analog computer produce a model for an unaggregated set of enzymes with given rate-parameters. Also, and more importantly, they provide information as to the kinetic behavior of a complex reacting with exogenous (i.e., "externalized") substrates in contrast to the actual situation in the overall reaction, which may involve endogenous or compartmentalized (i.e., "internalized") substrates.

transients, would only further validate our results and the conclusion that enzyme aggregates are, in actuality, integrated units of catalytic action. Additional analysis of the type conducted here in conjunction with direct measurements of the intermediate substrates generated during the course of the overall reaction, as has been done for the carbamyl phosphate synthase (22, 24) and tryptophan synthase (23) systems, should help resolve this problem.

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