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Role of Transcriptional Regulation and Enzyme Inactivation in the Synthesis of *Escherichia coli* Carbamoylphosphate Synthase

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The question of posttranscriptional control during cumulative repression of *Escherichia coli* carbamoylphosphate synthase has been examined by following the kinetics of repression and by comparing messenger ribonucleic acid and enzyme levels after growth under various conditions. The data provide no evidence for control of synthesis at a level other than transcription. They suggest, however, that enzyme inactivation (or turnover) plays a significant role in the establishment of repressive conditions.

Extensive data demonstrate control of bacterial enzyme synthesis at the transcriptional level. In contrast, the possibility that posttranscriptional regulation of gene expression also occurs remains a debated question. In the particular case of the arginine pathway of *Escherichia coli*, this question has been approached in several ways. A recent review summarizes and discusses the results obtained during these studies (19).

In this work, we examine the case of carbamoylphosphate synthase (EC 2.7.2.9). This enzyme, coded by the *carAB* gene cluster (12), is subject to cumulative repression by arginine and the pyrimidines (14, 16). Whereas limited information is available concerning the pyrimidine-mediated repression of this enzyme (13, 15), the participation of the arginine repressor in cumulative repression has been clearly established (10, 15). We now report the results of experiments designed to evaluate the role of posttranscriptional control in the regulation of the expression of the *car* genes.

The first experiment utilizes the kinetic approach that led Lavallé (7) to suggest that addition of arginine to the growth medium transiently inhibits the synthesis of the enzymes of this pathway. An arginine-repressible molecule, possibly an enzyme of the arginine pathway, was assumed to mediate this effect by interfering with the reading of the corresponding mRNA (see also Krzyzek and Rogers [6]). The variation of carbamoylphosphate synthase activity was followed over seven generations after the addition of arginine and uracil to the growth medium. Figure 1A shows the variation of specific enzyme activity. In Fig. 1B, the same values have been transformed into the relative increase of the enzyme content per milliliter of culture. In each figure, the experimental curve appears markedly different from the theoretical one calculated on the basis of exponential dilution of preexisting enzyme. Figure 1B indicates a pronounced drop in carbamoylphosphate synthase content after addition of arginine and uracil. This behavior appears different from those reported for *N*-α-acetylornithinase and ornithine carbamoyltransferase (7) or for tryptophan synthetase (8). The data cannot be explained solely by a transient arrest of enzyme synthesis after addition of end products, but rather, they suggest that an appreciable inactivation of carbamoylphosphate synthase takes place. Similar observations have been made for aspartokinase *I*-homoserine dehydrogenase I (2) and, more recently, for several arginine enzymes, including *N*-α-acetylornithinase (A. Boyen, Ph.D. thesis, University of Brussels, Brussels, Belgium, 1978). They suggest that enzyme inactivation plays a significant role in the elimination of enzyme surplus during the establishment of repressive conditions. The present case is particularly interesting since, as shown in Fig. 1, the experimental curves are fully accounted for, without the need for a transient regulation mechanism, by assuming a 20% inactivation of the enzyme per hour. This phenomenon may, however, not always be as obvious as it is here. In Fig. 2, we present a set of theoretical curves based on different rates of decay of carbamoylphosphate synthase activity: the inactivation is not very apparent up to 10% activity loss per hour. Such curves might thus be taken as evidence for a reduction in translational efficiency during the establishment of repression.

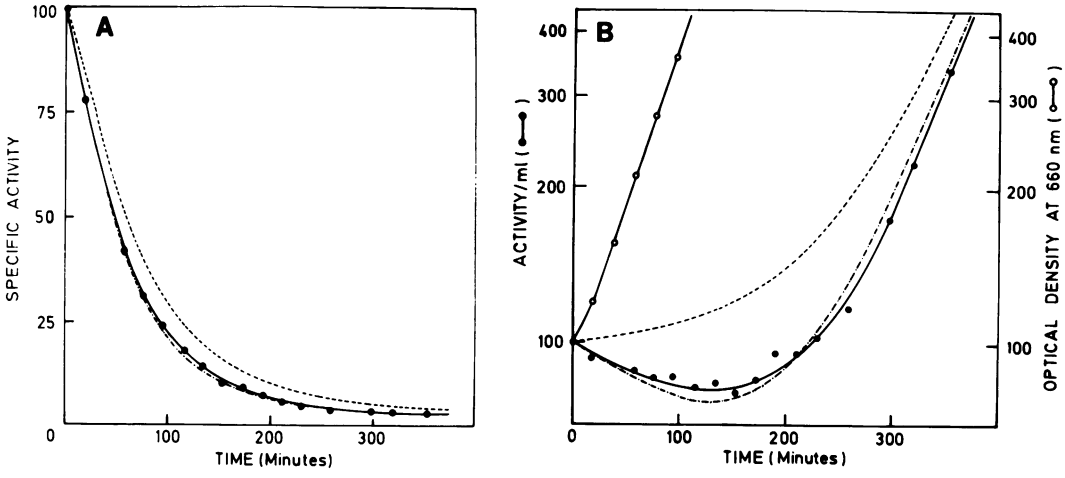

FIG. 1. *Kinetics of carbamoylphosphate synthase repression. Arginine (100 μg/ml) and uracil (50 μg/ml) were added at zero time to a 350-ml culture of the E. coli K-12 strain Hfr P4X (metB) growing exponentially on a rotary shaker at 37°C in minimal medium no. 132 (4) supplemented with 0.5% glucose and 50 μg of* L-*methionine per ml. Samples were removed from the culture at time zero and every 20 min and assayed for carbamoylphosphate synthase activity (15). The samples were replaced by an equivalent volume of fresh medium; optical density of the culture was maintained between 0.3 and 0.4. Protein content was estimated by the method of Lowry et al. (11). Specific activities are expressed as units (U; micromoles of carbamoylphosphate formed per hour per milligram of protein). Optical densities were determined using a Beckman B spectrophotometer. (A) Variation with time of the specific carbamoylphosphate synthase activity. Values are expressed as percent of the specific activity at zero time (1.008 U/mg of protein). The repressed activity was 0.034 U/mg of protein. The optical density at zero time was 0.303. The experimental curves are compared to theoretical ones calculated on the basis of exponential dilution of preexisting enzyme. These theoretical curves were calculated by introducing inactivation rates into the equation used by Lavallé and De Hauwer (8):*

$$A_{t'} = A_t + \frac{A_t - A_R}{2^{(t'-t)/g}} \times IR$$

where A_t is the specific activity at time t; $A_{t'}$ is the specific activity at time t'; A_R is the specific activity in the repressed state; $t' - t$ is the time elapsed from t to t'; g is the generation time in minutes; and IR is a factor which represents the fractional loss of enzyme activity during the period from t to t'. (-----) Theoretical curve based on exponential dilution of the preexisting enzyme accompanied by synthesis of new enzyme at the repressed rate of enzyme synthesis; (-·-·-·) theoretical curve calculated as above but assuming a 20% inactivation of carbamoylphosphate synthase per hour. (B) Experimental data from (A) plotted as the variation of carbamoylphosphate synthase per milliliter of culture. The values have been calculated by multiplying the specific enzyme activity at a given time by the optical density at that time and are expressed as percent of the content at zero times. Optical densities were corrected for successive dilutions of the culture. Symbols are as in (A).

Our attempts to characterize this inactivation of carbamoylphosphate synthase more precisely have so far been unsuccessful. We consequently do not know at present whether it corresponds to a specific inactivation mechanism or to a more general proteolytic degradative process. In any case, considering its metabolic position, the rapid disappearance of carbamoylphosphate synthase activity may offer a significant regulatory advantage during the installation of repression.

In a second approach, we have measured the *car* mRNA and carbamoylphosphate synthase levels after growth under various steady-state conditions of repression by arginine and uracil. As shown in Table 1, enzyme and mRNA were found to vary in a closely parallel manner over most of the range explored. Discrepancies such as those reported by Cunin et al. (3), who measured a total amplitude of variation three to four times lower for *argE* and *argCBH* mRNA than for the corresponding enzymes, were not observed. Such discrepancies have also been reported by Lavallé and De Hauwer (8) in the case of the tryptophan operon and confirmed by Krzyzek and Rogers (6) for the *argECBH* cluster. They have been interpreted in terms of their possible relation to translational control at the level of reading efficiency or of mRNA degradation. Cunin et al. (3) and Krzyzek and Rogers

(6) have pointed out that their observations could also be interpreted in terms of a larger participation in repression than in derepression of a normally untranslated segment of mRNA, in analogy with the situation described as "attenuation control" in the case of the tryptophan operon (1, 9). Our results thus provide no evidence for the participation of any of such mechanisms in the control of the *carAB* operon. A certain deviation from proportionality was apparent for the repressed level (Table 1), but went in a direction opposite to that observed for the arginine or tryptophan enzymes. This observation may lack significance, however, since messenger determinations were not more than 30% accurate at this level. There is thus certainly no suggestion that translation is less efficient at lower RNA levels. To establish a significant discrepancy in the opposite direction, which would be without precedent in our present state of knowledge, would require more accurate estimates.

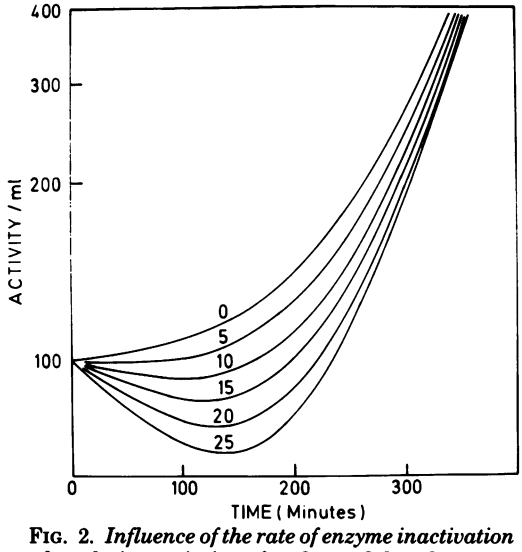

FIG. 2. *Influence of the rate of enzyme inactivation on the relative variation of carbamoylphosphate synthase content per milliliter of culture. A theoretical curve calculated as in Fig. 1B has been modified for various values of percentage of carbamoylphosphate synthase inactivation per hour (see indications on the figure). All other parameters used in the calculation are those of the experimental curves of Fig. 1.*

In conclusion, our kinetic and steady-state investigations of *carAB* gene expression provide no evidence for a posttranscriptional control. The parallelism observed between the variations of mRNA and of carbamoylphosphate synthase levels do not suggest a second-site transcriptional regulation either. More importantly, the kinetic data call attention to enzyme inactivation as a significant factor in establishing the balance of enzyme content. The latter phenomenon can mimic a transient translational control and seriously complicate analysis.

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TABLE 1. *Comparison of carAB mRNA and carbamoylphosphate synthase levels after growth under various conditions*[a]

Source of RNA	Addition to minimal medium[b]	Hybridization %[c]	Ratio[d]	Carbamoylphosphate synthase sp act[e]	Ratio[d]
P4XB2 λ⁻	None	0.437	1	2.35	1
P4X λ⁻	None	0.190	2.3	1.06	2.2
	Arginine	0.130	3.4	0.62	3.8
	Uracil	0.105	4.2	0.49	4.8
	Arginine + uracil	0.010	43.7	0.09	26.1

[a] Cultures (80 ml) of strain P4X λ⁻, cured of its original prophage, and of its derivative P4XB2 λ⁻(*metB argR*), genetically derepressed for the arginine enzymes, were grown exponentially in minimal medium to a density of 4×10^8 cells per ml. At this stage, 50 ml was used to determine carbamoylphosphate synthase specific activity, and the remaining 30 ml was submitted to pulse labeling with 1 to 4 mCi of [^{3}H]adenine (20 to 25 mCi/mmol; The Radiochemical Centre, Amersham, England) for 50 s. Conditions for RNA labeling and extraction were as described by Cunin et al. (3). Preparations of *l*-strands of the DNA of λd*carAB*37.9 and λ199 were as described by Lissens et al. (10). RNA hybridization assays were performed as described previously (17). The DNA from λd*carAB*37.9 is an excellent probe for *car* mRNA determination since it contains very little bacterial DNA in addition to the *car* genes (5), and low background values were consequently expected; this phage harbors 5.1 kilobase pairs of chromosomal DNA, of which 4.7 can be accounted for on the basis of the molecular weight of the enzyme (18), not including the control region.

[b] Arginine, 100 μg/ml; uracil, 50 μg/ml.

[c] Hybridization on *l*-λd*carAB*. The values were averaged from four or more determinations (in some cases, on samples from two independent cultures), each one performed in duplicate with at least 2 RNA concentrations. They were corrected for nonspecific hybridization to *l*-λ199. The counts of samples varied from 13,000 cpm for strain P4XB2 λ⁻ to 300 cpm for P4X λ⁻ grown on minimal medium plus arginine plus uracil.

[d] For each condition, the ratio was obtained by dividing the value for P4XB2 λ⁻ by the corresponding value.

[e] Micromoles of carbamoylphosphate formed per hour per milligram of protein.

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