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L-Arabinose Isomerase Formation in a Conditional Mutant of Gene *araA* of *Escherichia coli* B/r

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A temperature-sensitive mutant of *Escherichia coli* in which the synthesis of L-arabinose isomerase is blocked during growth at 42 C was found to possess the following properties. (i) The mutation occurred in the structural gene for the isomerase, gene *araA*. (ii) During growth at elevated temperatures the mutant accumulates a product which is a precursor to the active enzyme. (iii) The precursor produced at 42 C is slowly converted to active enzyme at 28 C in the absence of protein and ribonucleic acid synthesis. It is concluded that the mutation results in a change in the structure of isomerase which causes formation of active enzyme to be thermolabile at a step beyond the level of translation.

The first intracellular step in the catabolism of L-arabinose by *Escherichia coli* is catalyzed by the enzyme L-arabinose isomerase (EC 5.3.1.4). The structural gene for this enzyme is *araA*. Expression of *araA* is coordinate with *araB* and *araD*, and the three together comprise all structural genes of the L-arabinose operon (4, 11, 12). The regulatory gene for the operon is *araC* (4), and control over operon expression takes place at two sites termed *araI* and *araO* (5). The regulatory gene codes for a protein that has an affinity for the natural inducer, L-arabinose, and an anti-inducer, D-fucose. This regulatory gene protein functions as a repressor in the absence of inducer and in the presence of D-fucose (1, 17). In addition to its repressor activity, the *araC* protein also serves as a positive controlling element for operon expression when it is associated with the inducer. This positive control complex between inducer and protein is called an activator because it is essential for the expression of the structural genes under its control (4, 6, 16). The details of how the activator affects operon expression have not been resolved.

This report is concerned with the analysis of a mutation which appears to be in the *araA* gene and which causes the formation of isomerase to be temperature sensitive. The study reveals that the thermosensitive step in the production of active enzyme can be overcome in the absence of both protein and ribonucleic acid (RNA) biosynthesis. Since it is known that the active form of the native enzyme is composed of six homogeneous polypeptides (14), it is possible that the assembly process necessary for the aggregation of the functional protein is thermolabile in the mutant strain.

MATERIALS AND METHODS

Bacterial strains and culture media. All bacterial strains used are derivatives of *E. coli* B/r, and their pertinent genotypes are shown in Table 1. The composition of all culture media was described previously (9). Abbreviations for culture media are: AL, minimal L-arabinose (0.2%) supplemented with L-leucine (40 mg/liter); MA, minimal L-arabinose; EMB, Levine eosin methylene blue agar without lactose and supplemented with L-arabinose (1.0%); CAA, minimal base plus Casamino Acids (1.0%). When cultures were to be induced prior to enzyme assays, L-arabinose (0.4%) was added to CAA.

Transductions. Phage P1*bt* was propagated and used for transductions as previously described (8).

Complementation tests. F′ *ara* matings were performed by the two following methods. (i) Exponential L-broth cultures of F′ and F^- strains were mixed in a ratio of 1:9 and diluted into an equal volume of L-broth. The mixture was incubated with occasional gentle agitation for 1 hr at 37 C. Portions were then spotted onto AL agar and EMB agar. (ii) A 0.2-ml portion of a late-log-phase L-broth culture of the F^- strain was spread onto AL agar. Once the agar appeared dry, small portions of mid-log-phase L-broth cultures of the F′ strains were spotted onto the lawns. In both tests, complementation was recorded

TABLE 1. *Strains of Escherichia coli B/r*

Genotype	F′ homogenote	F⁻ haploid	Source
ara⁺ *leu*		*	E. Englesberg
araA35	*	*	E. Englesberg
araA54	*	*	E. Englesberg
araB24	*	*	E. Englesberg
araB71	*	*	E. Englesberg
araCOdel719[a]	*	*	E. Englesberg
araCdel766[a]	*	*	E. Englesberg
araD139 leu		*	E. Englesberg
araC42 leu		*	E. Englesberg
araC5 leu		*	E. Englesberg
araD139 araA307(TS) *leu*		*	This paper
araA307(*TS*) *leu*		*	This paper

[a] *araCOdel719* is a deletion of the operator site and of all of the *araC* gene. *araCdel766* is a deletion of about one-half of the *araC* gene distal from *araB* (5).

when confluent growth within the spot occurred in less than 30 hr of incubation of the agar plates at the desired temperature (16).

Enzyme assays. L-Arabinose isomerase was assayed by the methods previously described (9) except that the reaction mixture was incubated at 30 C rather than at 37 C. Under these conditions the L-ribulose production was linear for at least 0.5 hr. Each reaction mixture contained in a total volume of 1.0 ml: glycylglycine (*p*H 7.6), 125 μmoles; L-arabinose, 150 μmoles; $MnCl_2$, 2.5 μmoles; enzyme; and water. The reaction was initiated by addition of the enzyme preparation to the reaction mixture at 30 C. One unit of activity equals 1 μmole of ribulose formed per hr.

L-Ribulokinase (EC 2.7.1.16) was assayed by the spectrophotometric method of Lee and Englesberg (11). The reaction was measured in a Gilford recording spectrophotometer with 10 mm cuvettes. A 0.9-ml portion of the reaction mixture described below was mixed with enzyme and water in a total volume of 1.0 ml. The mixture was prepared by adding components in the order listed: water; reduced nicotinamide adenine dinucleotide ($NADH_2$), 1.5 mg; Sigma type I crude rabbit muscle preparation of pyruvate kinase and lactic dehydrogenase, 50 units of the former and 75 units of the latter; reduced glutathione, 6.0 μmoles; ethylenediaminetetraacetic acid (EDTA), 5.0 μmoles; $MgCl_2$, 50 μmoles; phosphoenolpyruvate, 25 μmoles; adenosine triphosphate, 10 μmoles; tris(hydroxymethyl)aminomethane-hydrochloride (*p*H 7.4), 450 μmoles; L-ribulose, 25 μmoles; final volume, 13.65 ml. A blank reaction mixture was also prepared without L-ribulose. The reaction was initiated by adding the enzyme preparation to the mixture at 25 C. Activity was determined from the difference in the initial velocities of the blank and L-ribulose-containing mixtures. One unit of activity equals 1 μmole of ribulose-5-PO_4 formed per hr, which is exactly equivalent to the oxidation of 1 μmole of $NADH_2$ per hr under these conditions.

Endogenous activities in the crude extracts were reduced to near zero by subjecting the crude preparation to centrifugation for 2 hr at 50,000 rev/min with a type 50 rotor in a Beckman L3-50 preparative ultracentrifuge.

Crude extracts were prepared for the enzyme assays from cultures grown in CAA medium. The cells were harvested from the culture medium by centrifugation at 10,000 × *g* in the cold, resuspended in an equivalent volume of 1.0 mM EDTA, and centrifuged again. The pellet of cells was then suspended in about one-fiftieth volume of 0.01 M glycylglycine buffer, *p*H 7.6. After four 10-sec sonic pulses, intact cells and debris were removed by centrifugation at 17,000 × *g* for 30 min in the cold.

Proteins were determined by the Lowry method (13).

Heat inactivation studies. Partially purified L-arabinose isomerase was studied for heat inactivation by previously described methods (15). Crude extracts were mixed with $MnCl_2$ (0.05 M final concentration) and held overnight in ice. After centrifugation at 10,000 × *g* for 10 min in the cold, the supernatant fraction was placed in a water bath at the desired temperature for heat inactivation. Portions of the heated extract were then removed and immediately added to isomerase reaction mixtures at 30 C which were then assayed for isomerase activity.

Capacity studies. Hartwell and Magasanik (7) showed it was possible to determine the relative level of messenger RNA (mRNA) for a specific gene product in a culture that was inhibited for RNA synthesis if conditions permitted translation of existing RNA species into protein. The specific activity of a given enzyme is determined before and after addition of an inhibitor of RNA synthesis. The difference in specific activities is termed capacity. One important assumption implicit in this type of test is that any increase in enzyme activity represents only newly synthesized enzyme. We find that L-arabinose isomerase capacity can be reproducibly measured in fully induced cultures.

Overnight cultures grown in CAA were diluted into fresh medium containing inducer. After 3 hr of growth at 28 C, the cells were collected by centrifugation at 10,000 × *g* for 1 min at 25 C. The pelleted cells were then resuspended in the same volume of culture medium prewarmed to 42 C and containing inducer. Growth was continued at 42 C. At times, two portions of the culture were taken. One was mixed with chloramphenicol (100 μg/ml) and immediately poured over crushed ice. The other was mixed with rifampin (54 μg/ml, a concentration found to inhibit at least 95% of RNA synthesis as measured by uridine incorporation into acid-precipitable material in these strains) to inhibit initiation of new RNA species, poured into a 250-ml flask, and incubated on a New Brunswick gyratory water bath shaker at 28 C for 45 min. After the incubation period, chloramphenicol was added, and the mixture was poured over crushed ice. Isomerase activity in both portions was determined, and the difference in activity in the two was

taken as the capacity to synthesize L-arabinose isomerase in the absence of mRNA synthesis. Capacity was calculated as: [(isomerase activity in the rifampin portion − isomerase activity in the untreated portion)/isomerase activity in the untreated portion] × 100.

RESULTS

Isolation of a temperature-sensitive mutant. Fifty 1-ml L-broth cultures of *araD139 leu* inoculated with fewer than 100 cells each were grown overnight at 37 C. Each culture was streaked onto EMB agar and incubated for 36 hr at 42 C. The growth of *araD139* is inhibited by arabinose under these conditions. Resistant clones arise which form large colorless colonies. These resistant clones result from second-site mutations in *araA*, *araB*, or *araC* (3, 10). Forty-two independent arabinose-resistant mutants were isolated at 42 C and tested for growth on EMB at 28 and 42 C. One formed large colonies at the high temperature and microcolonies characteristic of the parental *araD139* strain at the low temperature.

This strain, designated *araD139 ara-307*(TS), was saved, for the mutation conferring resistance appeared to be temperature sensitive. The other forty-one mutants formed resistant type colonies on EMB agar at both temperatures and were thus discarded.

The *araD139* mutant site was removed from the *araD139 ara-307*(TS) strain by transduction with P1*bt* phage grown on an *ara*$^+$ *leu*$^+$ strain. Transductants which were Ara$^+$ at 28 C were selected on AL agar. Transductants were then scored for leucine requirement and for temperature sensitivity of growth on L-arabinose. A leucine-requiring clone capable of growing on arabinose at 28 C but not at 42 C was purified and designated *ara-307*(TS) *leu*.

Enzyme activities in ara-307(TS). The wild type *ara*$^+$*leu* and *ara-307*(TS) were induced for 3 hr while growing at both 28 and 42 C in CAA medium and assayed for isomerase and kinase activities (Table 2). The wild-type strain had the expected levels of isomerase and kinase under the two growth conditions. The *ara-307*(TS) strain produced a lower level of isomerase at 28 C and almost undetectable isomerase at 42 C yet had similar kinase activities at both temperatures. This suggests that *ara-307*(TS) bears a mutation in the structural gene for the isomerase, *araA*.

Complementation tests. To confirm the assignment of the mutant site in *ara-307*(TS) to the *araA* gene, complementation tests were performed with the F′ homogenotes listed in Table 1. When the tests were performed with the single-site mutant strain, the spot plates were incubated at 42 C. Unexpectedly the single-site mutant strain complemented all of the F′ strains including F′*araA35/araA35* and F′*araA54/araA54*.

TABLE 2. *Levels of* L-*arabinose isomerase and* L-*ribulokinase*[a]

Strain	Growth temp (C)	Specific activities	
		Isomerase	Kinase
araA307(TS)	28	19.5	23.8
araA307(TS)	42	1.4	29.5
ara$^+$	28	50.1	26.2
ara$^+$	42	51.2	27.6

[a] Cultures were grown at the indicated temperatures in CAA medium containing inducer for 3 hr. Inocula were one-tenth volume of cultures grown overnight in CAA medium at the indicated temperatures.

The complementation tests were then repeated with the *araD139 ara-307*(TS) double mutant strain which is Ara$^-$ at both 28 and 42 C. The spot plates were incubated at 28 C, and all of the F′ homogenotes were complemented by the double mutant. The spots were then replica-plated to duplicate AL agar, and one was incubated at 42 C and the other at 28 C. Growth was observed within the area of the spots formed from the matings of F′*araB* and F′*araC* homogenotes with the double mutant at both temperatures. No growth at 42 C was observed within the spots formed from the matings with F′*araA35* and F′*araA54* homogenotes with the double mutant (Table 3). Cells growing in the spots on the 28 C replica plate were streaked to EMB agar and incubated at 42 C for 24 hr. Sectored (Ara$^+$ and Ara$^-$) colonies were detected in each of the isolates. This trait is a characteristic of complementing partial diploids (16). Cells from an Ara$^+$ portion of a sectoring colony were purified on EMB. In this way the merodiploids F′*araA54/araD139ara-307*(TS) and F′*araOCdel*719/*araD139ara-307*(TS) were formed.

These diploids, with appropriate haploid controls, were then grown and induced at 42 C and subsequently assayed for isomerase activity. The partial diploid F′*araA54/araD139 ara-307*(TS) had very low isomerase activity, just as the haploid *araA54* and *araD139 ara-307*(TS) strains produced low isomerase activities. On the other hand, F′*araOCdel*719/*araD139 ara-307*(TS) had high isomerase activity, and the haploid controls were deficient in isomerase, as expected (Table 4). We conclude, therefore, that the mutation which gave rise to the

TABLE 3. *Spot tests for complementation between F' homogenotes and F⁻ recipients*

F' Donors	28 C[a]				42 C[b]
	araD139 ara-307 (TS)	*araA35*	*araB24*	*araC5*	*araD139 ara-307* (TS)
araA35	C[c]	–	C	C	–
araA54	C	–	C	C	–
araB24	C	C	–	C	C
araB71	C	C	–	C	C
araOCdel719	C	C	C	–	C
araCdel766	C	C	C	–	C

[a] Temperature of incubation of the spot tests.

[b] Temperature of incubation of a replica plate of the spot tests performed at 28 C on AL agar. All spots showed growth within 16 hr on a control replica plate incubated at 28 C.

[c] C, Confluent growth on AL agar and black color formation on EMB agar in less than 30 hr. –, No growth on AL agar and pink or colorless spot on EMB agar after 30 hr.

TABLE 4. *L-Arabinose isomerase activity in merodiploids*

Exogenote	Endogenote	Isomerase (units per mg protein)
araA35	*araD139 araA307*(TS)	1.5[a]
araOCdel719	*araD139 araA307*(TS)	13.5[a]
araA35	*araA35*	0.1
araOCdel719	*araOCdel719*	0.1
	araD139 araA307(TS)[b]	0.7

[a] Portions of the cultures were streaked onto EMB agar for growth at 42 C to estimate stability of the heterogenotes. Greater than 95% of the resultant colonies were Ara⁺, less than 1% were Ara⁻, and the rest were sectored colonies.

[b] Haploid control.

araD139 ara-307(TS) double mutant strain occurred in gene *araA*. The mutant is hence designated as *araA307*(TS). An additional proof in support of this conclusion will be presented below.

Heat inactivation of L-arabinose isomerase. Since *araA307*(TS) fails to produce high levels of isomerase during growth at 42 C and yields 40% of the wild-type level at 28 C, it is possible to study various properties of the mutant enzyme in this strain by inducing isomerase at low growth temperatures. To test the possibility that the isomerase in *araA307*(TS) is thermolabile at 42 C, we compared its stability at 42 C to that of the wild-type enzyme both in vivo and in vitro and found no inactivation of either enzyme. Indeed, after 24 min at 50.5 C in vitro, both the wild-type and mutant enzymes retained 95% of their activities. At 58 C, however, the enzyme from *araA307*(TS) turned out to be much more sensitive than the wild-type isomerase (Fig. 1). This heat lability supports the notion that the mutation giving rise to *araA307*(TS) affected the structural gene for L-arabinose isomerase, but it does not account for the failure to detect enzyme activity in cultures of *araA307*(TS) induced at 42 C.

Because the experiment described above indicates that isomerase produced in *araA307*(TS) is relatively stable at 42 C, we next tried to demonstrate that the synthesis of isomerase is temperature sensitive. Cultures of both *araA307*(TS) and the wild type were induced for 3 hr while growing at 42 C. At that time, chloramphenicol (100 μg/ml final concentration) was added, and the cultures were divided into 50-ml portions which were incubated in a shaking water bath equilibrated at 28 C. At the desired times flasks were removed, and the cell contents were assayed for isomerase activity. Isomerase activity increased with time in *araA307*(TS) and remained relatively constant in the wild-type control (Fig. 2). This

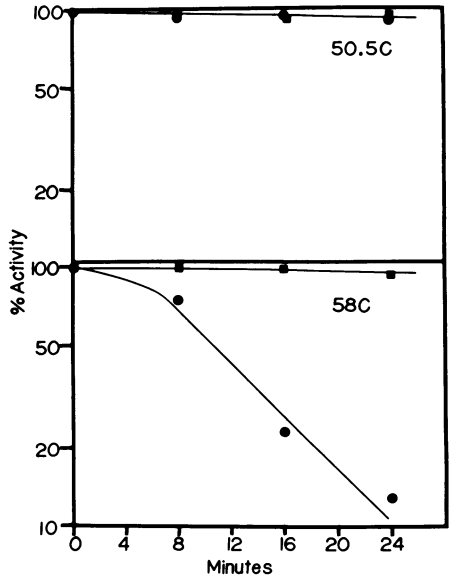

FIG. 1. *Heat inactivation of L-arabinose isomerase. Cultures of araA307 (TS) and ara⁺ were induced during growth in CAA medium at 28 C. Crude extracts were precipitated overnight with 0.05 M $MnCl_2$, and the supernatant fractions were incubated at the indicated temperatures to determine isomerase stability. Solid squares, ara⁺; solid circles, araA307 (TS).*

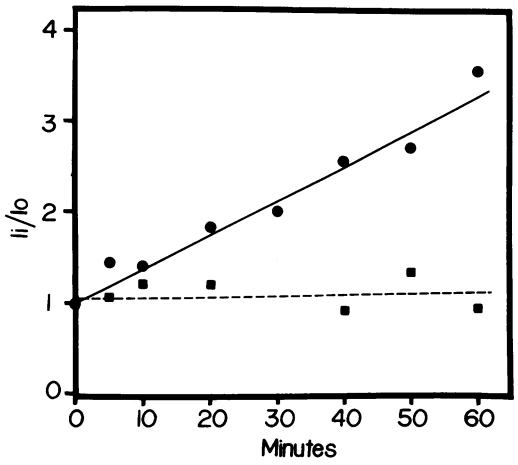

FIG. 2. *Levels of* L-*arabinose isomerase during incubation with chloramphenicol at 28 C. Cultures of araA307 (TS) and ara+ were induced for 3 hr during growth at 42 C. At zero time chloramphenicol was added at 100 μg/ml, and the cultures were shifted to 28 C. At the indicated times, portions were assayed for isomerase activity.* I_i/I_o = *isomerase activity at a given time* (T_i) *divided by isomerase activity at zero time. Solid squares, ara+; solid circles, araA307(TS).*

finding suggests that *araA307*(TS) forms a precursor to isomerase during growth at 42 C that is slowly converted to active isomerase at 28 C. A comparable isomerase precursor is not detectable in the wild-type strain. Since this precursor is converted to functional enzyme without protein synthesis, it seems likely that it is the *araA* gene protein in an inactive state.

The isomerase activities in the crude extracts from the above experiment were each tested for their thermal stability by incubation of the supernatant fluid obtained after $MnCl_2$ precipitation. Two assays were run; one before heating and one after heating at 58 C for 16 min. Each extract from *araA307*(TS) had approximately 50% isomerase activity remaining after the heat treatment, and the wild-type extracts showed little change in activity. Thus, the active enzyme produced in the above manner in the mutant strain has a thermal stability quite similar to the enzyme synthesized during growth at 28 C.

The kinetics of accumulation of the isomerase precursor formed at 42 C in *araA307*(TS) can be estimated from an experiment meant to measure L-arabinose isomerase capacity, i.e., the level of mRNA specific for L-arabinose isomerase. The capacity assay depends upon the assumption that any active enzyme which accumulates after the inhibition of RNA synthesis represents the translation of pre-existing pools of the specific mRNA. Reasonable estimates of isomerase mRNA can be made in this way in strains which have no apparent precursors for isomerase present. In the case of *araA307*(TS), however, the assay apparently reflects the amount of mRNA plus the amount of enzyme precursor.

When the wild-type strain was assayed for isomerase capacity, relatively constant levels of newly synthesized enzyme were detected at different periods of time after the temperature shift (Fig. 3). Capacity levels were also determined in a temperature-sensitive mutant that maps in the regulatory gene of the arabinose operon. This is mutant *araC42* which produces a functional, positively acting, regulatory gene protein at 28 C but lacks this activity at 42 C (9). In this strain, isomerase capacity rapidly decayed during growth at 42 C, indicating that no new mRNA was being synthesized at this temperature because of the thermolability of the regulatory gene protein. When the test was performed with *araA307*(TS) isomerase capacity increased continuously for at least one generation of growth at 42 C. Because inactive isomerase exists in cultures of *araA307*(TS) grown at 42 C, then this capacity represents

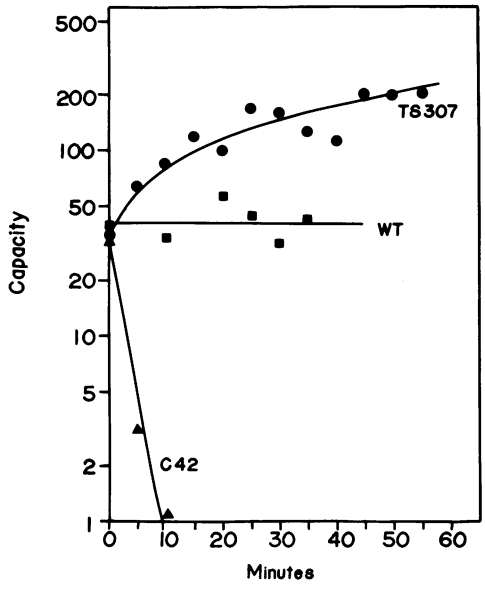

FIG. 3. L-*Arabinose isomerase capacity at 42 C. Cultures of ara+, araA307(TS), and araC42 were induced for 3 hr during growth at 28 C and were shifted to 42 C. At the indicated times, capacity for* L-*arabinose isomerase was determined. Capacity units and detailed procedures are described in Materials and Methods. Solid circles, araA307(TS); solid squares, ara+; solid triangles, araC42.*

both the accumulation of isomerase precursors and the level of existing mRNA in this mutant strain.

DISCUSSION

Early studies reported unambiguously that the *araA* gene is the structural gene for L-arabinose isomerase (2, 11). Since *araA307*(TS) fails to yield isomerase activity during growth at 42 C in the haploid state and in merodiploids with authentic *araA* mutants, and because *araA307*(TS) produces isomerase at 28 C which is more thermolabile than the wild-type enzyme at 58 C, it is concluded that *araA307*(TS) is a mutation of the *araA* gene. The fact that the single-site mutant separated from *araD139* gave a positive response in the usual complementation tests is not understood, and any explanations would be mere suppositions.

The isomerase has been purified from *E. coli* and found to exist as a protein with a molecular weight of 362,000 which can be dissociated into homogeneous subunits by protonation at *p*H 2.0 or by treatment with 8 M urea. The subunits have a molecular weight of 60,000, which suggests that the native enzyme is composed of six identical monomers (14). In additional studies, Patrick et al. (15) found that the intrinsic activity of isomerase was not always the same from culture to culture even though the levels of enzyme protein were comparable when tested against anti-isomerase serum. The difference in intrinsic activity was attributed to a dependence upon manganous ions during synthesis of the enzyme at some step beyond the level of translation. This finding, together with the fact that the active enzyme is a hexamer, suggests that there is a critical process beyond translation necessary for the formation of active isomerase.

Properties of the isomerase produced in *araA307*(TS) are compatible with the view that one or more steps for the production of active isomerase occur beyond the level of translation. Presumably one step involves the assembly of the monomers encoded by the *araA* gene. The simplest interpretation is that the mutation which gave rise to *araA307*(TS) results in the synthesis of monomers which can not participate in the assembly process during growth at elevated temperatures. Upon lowering of the temperature of incubation of the mutant, there must be sufficient refolding of the peptides to allow slow conversion to the active form of the enzyme in its hexameric state. Whether the protein synthesized in *araA307*(TS) at 42 C remains as a monomer or whether it aggregates into some higher level of organization is not yet known but can be studied. It seems, therefore, that this temperature-sensitive mutant may serve as a valuable tool for the study of the process of protein-protein interactions because the assembly process can be controlled by an easily manipulated parameter.

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