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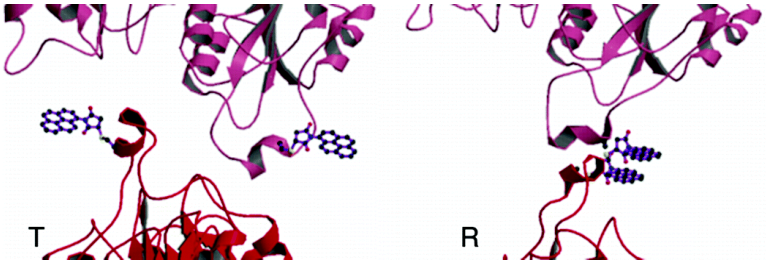

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Trapping Specific Quaternary States of the Allosteric Enzyme Aspartate Transcarbamoylase in Silica Matrix Sol−Gels

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Significant structural rearrangements are necessary for the function of many proteins, and in particular multisubunit allosteric enzymes involved in the regulation of metabolic pathways. These enzymes usually exist in at least two structural and functional states, T and R, in equilibrium. The T state has low substrate affinity and/or catalytic activity, while the R state has high substrate affinity and/or catalytic activity; however, the independent characterization of these two functional states has not been possible. Here we use a combination of a pyrene-labeled enzyme and encapsulation in sol−gels as a means to slow the interconversion of the two forms sufficiently so that either can be studied independently, as well as the transition between the two forms.

Aspartate transcarbamoylase (ATCase) from *Escherichia coli* is a dodecameric allosteric enzyme (M_r 310 000) that utilizes six active sites to catalyzes the first step in the pyrimidine biosynthesis pathway, the condensation of L-aspartate and carbamoyl phosphate, and utilizes six control sites to regulate the flux of metabolites through this pathway. The end products of the pyrimidine pathway CTP and UTP are allosteric inhibitors[1] while ATP, the end product of the parallel purine pathway, is an allosteric activator.[1] The R quaternary structure can be cooperatively induced by addition of substrates and/or substrate analogues, such as the bisubstrate analogue *N*-phosphonacetyl-L-aspartate (PALA), allowing the X-ray structures of both states to be determined to atomic resolution.[2] The kinetics of the allosteric transition in ATCase has been studied by time-resolved stopped-flow small-angle X-ray scattering (SAXS). The evolution from the T quaternary structure to the R quaternary structure was monitored after mixing the enzyme with the substrates,[3] or with carbamoyl phosphate and the aspartate analogue succinate.[4] With the experimental equipment available, complete scattering curves could not be collected at rates faster than every 100 ms, and cryogenic conditions had to be employed (temperatures between −11 and −5 °C using 30% ethylene glycol in the buffer) because of the rapid rate of the allosteric transition. To monitor the quaternary structure change under more physiological conditions, we have developed a pyrene-labeled ATCase, the fluorescence of which is sensitive to changes in the quaternary structure of the enzyme.[5] As shown in Figure 1, in the T quaternary structure the pyrenes are far apart and therefore only show monomer fluorescence (380 nm), while in the R quaternary structure the pyrenes are close together exhibiting excimer fluorescence (480 nm). This pyrene-labeled ATCase provides a direct means to determine the quaternary conformation of the enzyme not only in solution but also in the confined matrix of a silica sol−gel.

A silica sol−gel is a transparent glass matrix formed by the polymerization of a silicon alkoxide. The matrix environment provides a convenient method to trap biological molecules and significantly inhibit and slow quaternary conformational changes,[6] while retaining catalytic activity by allowing the rapid transport of small molecules such as substrates and products.[7] For hemoglobin[8] and transferrin,[9] the rates of the quaternary change within the sol−gel have been studied. We show the feasibility of measuring the reaction kinetics of the isolated T and R states, as well as measuring the kinetics of the allosteric transition on a much slower time scale than in solution, using pyrene-labeled ATCase in hydrated sol−gels.

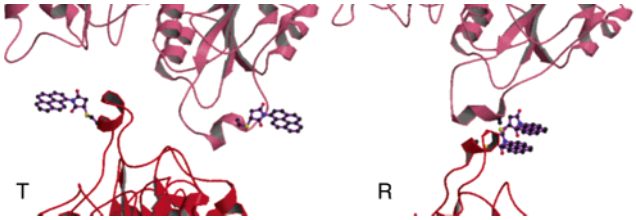

Figure 1. Model of the loop region of ATCase labeled with pyrene in the T and R states. Excimer formation is only possible in the R state.

The encapsulation of ATCase was carried out by first making a stock solution of acidified tetramethoxysilane (TMOS), prepared by mixing TMOS and 0.002 M hydrochloric acid in a 1:2 ratio with vigorous stirring at room temperature until the solution was monophasic (5−10 min). For the enzyme-doped sol−gels used in the kinetic experiments, 0.030 mL of the TMOS sol was mixed with 0.02 mL of a 0.015 mg/mL solution of ATCase in 50 mM Tris-acetate buffer, pH 8.3, in a rotating glass tube (1 cm diameter). The gel formed within 2 min at room temperature. The 50 mM Tris-acetate buffer was sufficient to ensure that the enzyme was not exposed to low pH during the polymerization. The resultant ATCase-doped thin film, which adhered to the inner surface of the glass tube, was soaked in 50 mM Tris-acetate buffer, pH 8.3, at 4 °C overnight before use. For the enzyme-doped sol−gels used in the fluorescence experiments, 1.5 mL of the TMOS sol was mixed with 1 mL of a 0.1 mg/mL pyrene-labeled ATCase in 50 mM Tris-acetate buffer, pH 8.3, in a methacrylate cuvette (1 cm path length). The resultant ATCase-doped sol−gel monolith was allowed to gel overnight at 4 °C and then soaked in 50 mM Tris-acetate buffer, pH 8.3, for storage before use.

Kinetics experiments (aspartate saturation curves using the colorimetric method[10] in 50 mM Tris-acetate buffer, pH 8.3) demonstrated that the wild-type and pyrene-labeled enzymes encapsulated in the thin film sol−gels retained catalytic activity, with a maximal activity approximately one-half that in solution (data not shown). However, the $[Asp]_{0.5}$ was significantly reduced for both enzymes as compared with the saturation curves performed in solution, and the saturation curves were hyperbolic for both enzymes as compared with the sigmoidal curves observed in solution. In addition, as shown in Figure 2A, fluorescence spectra of the sol−gel monoliths demonstrated that the pyrene-labeled enzyme encapsulated without ligands had an emission spectra very similar to the enzyme encapsulated with a saturating concentration of PALA (R state control). This result was unexpected. An estimate of the allosteric equilibrium constant, L (as defined by the Monod Wyman Changeux Model,[11] L = [T state]/[R state]) for ATCase is

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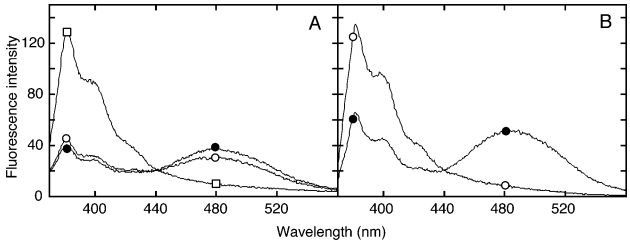

Figure 2. Fluorescence emission spectra for the sol−gel encapsulated (A) and unencapsulated pyrene-enzyme ATCase in solution (B). The emission curves are shown for the enzyme without ligands (○), with a saturating concentration of PALA (●), and with a saturating concentration of CTP (2 mM) and UTP (2 mM) (□). All fluorescence spectra were recorded at 25 °C in 50 mM Tris-acetate buffer, pH 8.3.

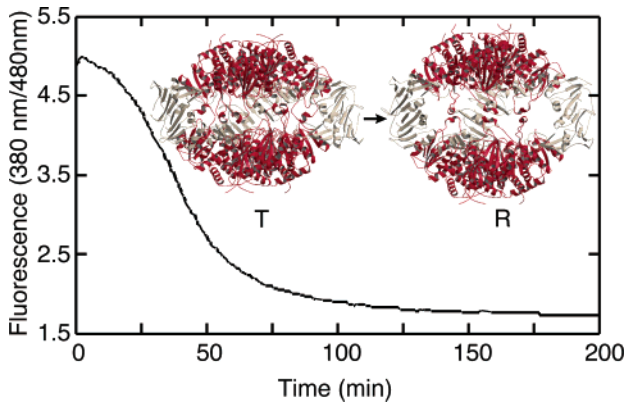

Figure 3. Time evolution of the T-to-R structural transition as observed by the change in the fluorescence emission spectra (peak ratio 380 nm/480 nm) for the sol−gel encapsulated pyrene-labeled ATCase after addition of a saturating concentration of PALA (2 mM). The fluorescence spectra were recorded at 25 °C in 50 mM Tris-acetate buffer, pH 8.3. Inset: X-ray structures of the T and R structural states of ATCase holoenzyme.[2]

approximately 250,[12] indicating that the T state is highly favored in the absence of substrates. To encapsulate the T state quaternary structure, the heterotropic inhibitors known to stabilize the T state, CTP and UTP, were added at saturating concentrations (2 mM) before encapsulation in the sol−gel monolith to trap the T state, and then rinsed several times with 50 mM Tris-acetate buffer, pH 8.3, after gelation to remove the residual nucleotides before use. Encapsulation of the T-state is demonstrated by a comparison of the fluorescence spectra of the enzyme encapsulated with CTP and UTP in the sol−gel (Figure 2A) with the enzyme in solution (Figure 2B), showing that they are identical. This indicates in the absence of ligands a mixture of the T and R quaternary structures are trapped in the sol−gel, with the R state being favored by a considerable amount. This result also explains the similar kinetics observed for enzyme encapsulated in the absence or presence of substrates and/or substrate analogues.

Figure 3 shows the kinetics of the T-to-R allosteric transition in ATCase, initiated by addition of a saturating concentration of PALA (2 mM) to the wet sol−gel monolith. The data in Figure 3 exhibit a pure first-order decay after an initial lag phase (0 to 30 min) for the T-to-R transition. We attribute the lag phage to equilibration of the PALA into the relatively thick sol−gel monolith. The first-order rate constant calculated from the data after the initial lag phase (0.039 min^{-1}), corresponds to a half-time of approximately 18 min. The experiment was also performed using a 5-fold higher concentration of PALA (10 mM). No change in the rate was observed (data not shown), indicating that the process was not pseudo-first order, and indicating that the observed change in fluorescence was reflecting the structural alteration of ATCase from the T to the R quaternary structure within the sol−gel. There was no evidence of any structural intermediates between the T and R states, which agrees with previous time-resolved SAXS experiments.[3,4] This does not rule out their existence, only that they cannot be observed by these techniques. Data were collected after the end of the data displayed in Figure 3. After 24 h, the fluorescence (380 nm/480 nm) was 1.15, close to the value of 1.05 observed in solution for the pyrene-labeled enzyme saturated with PALA. The rate of the allosteric transition in the sol−gel matrix can be compared to the rate in solution as determined by fluorescence stopped-flow of the pyrene-labeled ATCase which is approximately 10^5 faster under similar conditions.

In conclusion, sol−gel encapsulation of ATCase dramatically slows the rate of the conversion of the enzyme from the T to the R structure. Furthermore, the regulatory inhibitors CTP and UTP are shown to stabilize the T quaternary structure of the enzyme. This technique not only yields a method of investigating the kinetics of the allosteric transition of ATCase but also the kinetic characterization of the individual quaternary structures of the enzyme. It should also be possible to determine if the allosteric effectors, such as ATP and CTP, have regulatory effects on the isolated T and R allosteric states.

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