Structural kinetics of the allosteric transition of aspartate transcarbamylase produced by physiological substrates

Hirotsugu Tsuruta[1], Takayuki Sano[1], Patrice Vachette[2], Patrick Tauc[3], Michael F. Moody[4], Katsuzo Wakabayashi[5], Yoshiyuki Amemiya[6], Kazumoto Kimura[7] and Hiroshi Kihara[8]

[1]*Department of Materials Science, Faculty of Science, Hiroshima University, Higashisenda, Hiroshima 730, Japan,* [2]*LURE, Bâtiment 209d, Université Paris-sud, F91405 Orsay Cedex, France,* [3]*Institut d'Enzymologie, CNRS F91190 Gif-sur-Yvette, France,* [4]*School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX,* [5]*Department of Biophysical Engineering, Faculty of Engineering Science, Osaka University, Machikaneyama, Toyonaka 560, Japan,* [6]*Photon Factory, National Laboratory for High Energy Physics Oho, Tsukuba 305, Japan,* [7]*Division of Medical Electronics, Dokkyo University, School of Medicine, Mibu, Tochigi 321-02 Japan* and [8]*Jichi Medical School, School of Nursing, Minamikawachi, Tochiga 329-04, Japan*

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We have studied the kinetics of the quaternary structure change associated with the allosteric transition of aspartate transcarbamylase (ATCase) (*E. coli*), inducing this change by exposure to the natural substrates (carbamyl phosphate and L-aspartate). The presence of 30% ethylene glycol slowed the quaternary structure change sufficiently for it to be followed by stopped-flow X-ray scattering at −5°C. After adding substrates to the enzyme, the change occurred, with a half-life of a few seconds, yielding a mixture of the two standard quaternary structures (or, conceivably, a state intermediate between them). This mixture persisted until the enzyme reduced the substrate concentration below a threshold value.

Allostery; Aspartate transcarbamylase; Fast kinetics; X-ray scattering

1. INTRODUCTION

Aspartate transcarbamylase (ATCase) catalyses the first committed step of pyrimidine biosynthesis, the reaction of L-aspartate with carbamyl phosphate. The enzyme is allosteric, showing homotropic cooperativity with substrates, and heterotropic effects with ATP or CTP. These properties are probably related to the existence of two quaternary structures: one found without ligands, or with CTP, and the other with substrate analogues or N-phosphonacetyl-L-aspartate (PALA). The quaternary structures are revealed as X-ray solution scattering patterns [3,6], which correlate well with the quaternary structures found by crystallography [1,2]. A programme of apparatus development [5–9] has finally produced a stopped-flow X-ray scattering device suitable for following these quaternary structure changes after enzyme is mixed with various substrate solutions, in the presence of ethylene glycol. In our first study [10], the reaction was slowed by using acetyl phosphate instead of the natural substrate, carbamyl phosphate. The rate of the structural change could be measured, and was roughly similar to the rate (measured by chemical quench) at which the enzymatic activity was switched on [10]. Recent improvements in the apparatus allow us to monitor the structural change induced by the natural substrates (at −5°C, in the presence of 30% ethylene glycol).

Correspondence address: H. Tsuruta, Department of Materials Science, Faculty of Science, Hiroshima University, Higashisenda, Hiroshima 730, Japan

2. MATERIALS AND METHODS

ATCase was prepared [11] from *E. coli*. It appeared homogeneous by polyacrylamide gel electrophoresis with sodium dodecyl sulphate, and showed only traces of dimer under non-denaturing conditions. The buffer used throughout the experiment contained 63 mM Tris, 56 mM borate, 0.07 mM ethylenediamine tetraacetate, 0.07 mM phenyl methyl sulphonyl fluoride, 0.07 mM dithiothreitol, at pH 8.3. All solutions contained 30% ethylene glycol. The enzyme solution was prepared by adding buffer (containing 60% ethylene glycol) to concentrated enzyme solution (about 150 mg/ml), until the mixture reached 30% ethylene glycol. The addition was performed gradually and with gentle stirring to avoid denaturation and precipitation of the enzyme. The substrate solution (containing carbamyl phosphate from Sigma) was prepared just before the experiment to minimise hydrolysis.

The time-resolved X-ray scattering experiment was carried out at the small-angle X-ray scattering installation (BL-15A) in the Photon Factory, National Laboratory for High Energy Physics, Tsukuba, Japan [12]. The storage ring was operated at 2.5 GeV with a ring current between 170 and 320 mA. The sample solution was irradiated with a monochromatic X-ray beam (1.50 Å wavelength, giving a beam at the sample position of 10^{11} photons/s and 1.8 × 1.9 mm^2). Scattered X-rays were recorded with a position-sensitive proportional counter 1.15 m from the sample. The average count rate was 18000 counts/s (from buffer) and 45000 counts/s (from enzyme solutions).

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Mixing was achieved with a stopped-flow device recently developed for use at subzero temperatures and with a dead time of 10 ms [13]. Scattering patterns were collected with the following time-slice schedule: first 25 frames of 0.1 s each, then 25 frames of 0.2 s each, then 20 frames of 0.4 s each, then 20 frames of 2.0 s each, then 5 frames of 5.0 s each; the entire schedule taking 80.5 s. All scattering data were normalised for incident beam intensity and counting time.

3. RESULTS AND DISCUSSION

Fig. 1 shows the scattering pattern (A) of the enzyme after mixing. Two quaternary structures' scattering curves are also represented: the unligated or 'T' in (B), and the PALA-ligated or 'R' in (C). An intermediate curve (like A) might result from scattering by a mixture of 'T' and 'R' quaternary structures. Making that assumption, intermediate scattering patterns were approximated by a linear combination of the 'T' and 'R' patterns, with the coefficients giving us the proportions of the two standard quaternary structures in the mixture. We found such linear combinations to fit the experimental curves satisfactorily, except (in some cases) for a very small difference around $s = 0.03$ Å^{-1}.

The time course of the reaction can be shown by plotting (as in Fig. 2) the scattered intensity integrated over a given range of s (s_1 to s_2 in Fig. 1). The early rising phase (Fig. 2) shows the induction of the 'R' quaternary structure by substrates. The rate constant for this transition rises with the initial aspartate concentration until the latter reaches 400 mM; it seems that the transition occurs more rapidly when more of the enzyme's sites bind substrate.

This rising phase is followed by a plateau, where the enzyme is presumably saturated with substrate. (However, the scattering curve does not then correspond to the PALA-saturated curve, but to an intermediate curve, as if even saturating concentrations of the natural substrates can only shift the 'T'⟶'R' equilibrium half-way to the 'R' form, at least in 30% ethylene glycol.*) This plateau phase lasts until the enzymatic reaction has reduced the aspartate concentration below some threshold value; then the scattering intensity falls slowly back to (nearly) its original value. Both the length of the plateau phase, and the slowness of the subsequent fall, are greater if higher initial aspartate concentrations are used. (Thus the total X-ray scattering, over this angular range and over all times subsequent to mixing, is proportional to the initial aspartate concentration.) This can be understood as follows. The time taken by the enzyme to reduce the

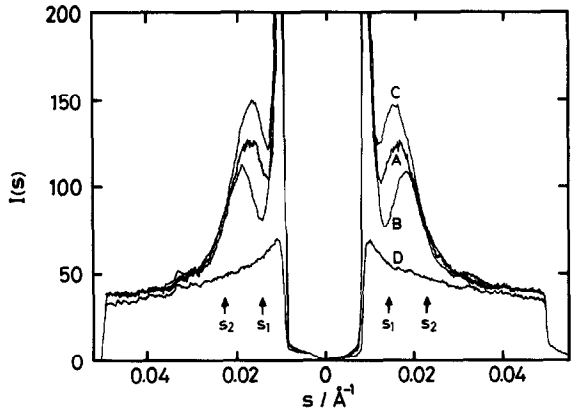

Fig. 1. A time-resolved X-ray solution scattering pattern of ATCase recorded during the reaction with native substrates, together with reference ATCase X-ray scattering patterns. The ATCase solution (76.5 mg/ml = 1.5 mM in active sites), containing 44 mM carbamyl phosphate in 30% ethylene glycol, was mixed with the substrate solution (400 mM aspartate and 44 mM carbamyl phosphate in 30% ethylene glycol). 0.04 ml of substrate solution were mixed (at −5°C) with 0.14 ml of ATCase solution. The pattern (A) represents the averaged data of 13 identical runs. Scattered counts are plotted against the reciprocal coordinate s ($=(2/\lambda)\sin\theta$, where 2θ is the scattering angle). s_1 and s_2 mark the values of the reciprocal coordinate used in Fig. 2. (A) The plateau phase (steady state) recorded 16.5 s after mixing; (B) ATCase (at the same concentration) without substrates or ligands; (C) ATCase (at the same concentration) saturated with PALA; (D) the background scattering.

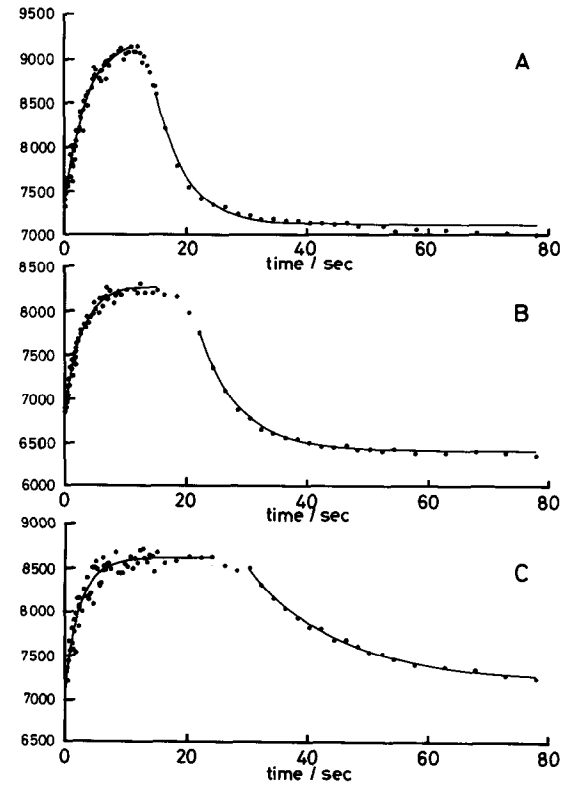

Fig. 2. Time course of the reaction with different aspartate concentrations after mixing: (A) 44 mM; (B) 89 mM; (C) 174 mM. (Other reaction conditions were the same as in Fig. 1.) The ordinate shows the total count integrated over the range s_1 to s_2 (shown in Fig. 1). Each continuous line shows a single exponential rise (or fall) with the rate constant adjusted to give a least-squares fit. The rate constants are: (A) 0.27 s^{-1} (rise) and 0.20 s^{-1} (fall); (B) 0.36 s^{-1} (rise) and 0.16 s^{-1} (fall); (C) 0.38 s^{-1} (rise) and 0.066 s^{-1} (fall).

* It cannot be excluded that, instead, the mixture consists entirely of a new quaternary structure, less expanded than that studied by crystallography [1,2], but yielding a scattering pattern that can be represented as a linear combination of the patterns of the standard 'T' and 'R' forms. It would be difficult to decide between these possibilities, unless ATCase saturated with aspartate and carbamyl phosphate (in ethylene glycol) could be crystallised.

aspartate concentration to the threshold value will determine the length of the plateau phase. Consequently that length will be proportional to the initial aspartate concentration, if the level of enzyme activity is constant during the plateau phase. Once the aspartate concentration has been reduced to the threshold value, the residual carbamyl phosphate concentration (and hence the rate of further substrate consumption) will be lower if more aspartate was present in the original mixture.

All these experiments show, at a level of detail unapproached by any other technique, the entire kinetics of the quaternary structure change of ATCase during its reaction with the natural substrates. The influence of allosteric effectors on the scattering patterns, and on the course of the reaction, is now being studied by stopped-flow X-ray scattering. In addition, chemical quench experiments will be performed under identical conditions to measure the rate constants for enzyme activation. Those experiments will provide kinetic tests of the various allosteric theories, such as the 2-state Monod-Wyman-Changeux model [14].

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