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Effects of Macromolecular Crowding on the Intrinsic Catalytic Efficiency and Structure of Enterobactin-Specific Isochorismate Synthase

Ming Jiang and Zhihong Guo*
Department of Chemistry, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong SAR, China.
Received July 16, 2006; E-mail: chguo@ust.hk

Enzymes play essential roles in all aspects of life processes, catalyzing biochemical reactions in a highly crowded environment where the total macromolecular concentration is 8−40%.[1] It is of intense interest to understand the effects of this high molecular crowdedness, termed macromolecular crowding, on enzyme catalysis. Through affecting macromolecular association and diffusion, macromolecular crowding has been demonstrated to significantly alter the activity of enzymes processing macromolecular[2] and small molecule[3] substrates. In addition, a recent computational study has shown that macromolecular crowding affects the internal enzyme dynamics and has a direct impact on enzyme catalysis.[4] However, no experimental report is available on whether and how the intrinsic catalytic capability of an enzyme is altered in a milieu similar to the natural enzyme environment, when the crowding-susceptible macromolecular association is not involved. Here we report that macromolecular crowding changes the structure and increases the intrinsic catalytic efficiency of the isochorismate synthase (EntC) specific for bacterial enterobactin biosynthesis in the absence of macromolecular association.

EntC is a monomeric enzyme catalyzing reversible conversion between chorismate and isochorismate in *Escherichia coli*.[5] By coupling to the reactions catalyzed by excess isochorismatase (EntB) and L-lactate dehydrogenase (LDH), the EntC-catalyzed forward reaction becomes virtually irreversible and is separated from the reverse reaction for study under crowding conditions. The equilibrium dissociation constant (K_D) of a potential EntC interaction with itself or either of the coupling enzymes was estimated to be greater than 100 μM by surface plasmon resonance (Figure S1, Supporting Information). In addition, a constant specific activity was found for EntC at a concentration from 10 nM to 4 μM (Figure S2), demonstrating that neither self-association of EntC nor its interaction with the coupling enzymes occurs to affect its activity over the 400-fold concentration range. Even under crowding conditions that can increase the equilibrium association constant by 40- to 80-fold,[1b,2b,6] it is not possible for EntC at nanomolar concentration to be involved in a detectable protein association because of the high K_D of all potential interactions. This provides an ideal system to observe the effects of macromolecular crowding on the intrinsic activity of EntC without the interference from macromolecular association.

The catalytic parameters of EntC at 30 nM were determined in a concentrated solution of Ficoll 70, a neutral polysaccharide recommended for mimicking the intracellular macromolecular crowding.[7] The crowding agent significantly decreases the K_M of EntC, particularly at low concentrations from 0 to 20%, and affects k_{cat} to a much less extent, resulting in a steady increase of catalytic efficiency (k_{cat}/K_M) (Figure 1). Similar rate enhancement was also observed in a solution of Dextran, another polysaccharide crowding agent. In contrast, no enhancement of k_{cat}/K_M was observed in sucrose solution, which is similar in polarity to a solution of either Ficoll 70 or Dextran at the same concentration. This control

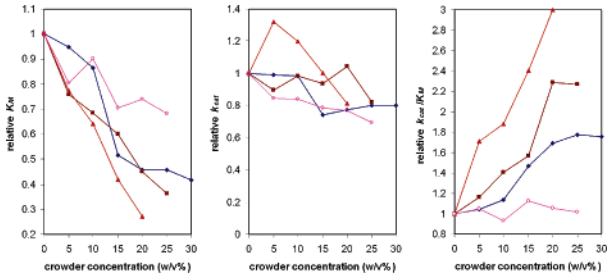

Figure 1. Kinetic parameters of EntC in solution of sucrose and three crowding agents: ◆, Ficoll 70; ■, Dextran; ▲, PEG6000; ○, sucrose. Each data point is an average of three independent measurements (Figure S3).

experiment with sucrose shows that the EntC rate enhancement is not due to crowding-induced polarity change.

In a solution of poly(ethylene glycol) (PEG6000), another commonly used crowding agent, K_M decrease and k_{cat}/K_M increase are similar to, but more pronounced than, that in Ficoll or Dextran solution (Figure 1). However, k_{cat} of EntC is significantly decreased after an abrupt increase at a low concentration, similar to the PEG effect on trypsin activity in a previous report.[8] This distinct k_{cat} effect is unlikely due to the nonspecific steric repulsion—the only influence exerted by macromolecular crowding—from PEG, but due to its known additional specific interaction with proteins.[2a,6b] This is supported by the observation of EntC precipitation in solution containing 25% or higher PEG6000. Despite this special PEG effect, the converging rate enhancement observed for all three crowding agents shows that macromolecular crowding enhances the intrinsic catalytic efficiency of EntC mainly by decreasing the Michaelis−Menten constant, K_M.

Similar K_M decrease has been previously observed in polymer cosolvents,[9] suggesting that it may occur in many other enzyme systems. To corroborate this, kinetic parameters were also determined for the following enzymic reactions in Ficoll solution: the forward reaction catalyzed by another monomeric isochorismate synthase (MenF),[10] EntB-catalyzed reaction, and pyruvate reduction by LDH. EntB[11] and LDH[12] are a homologous trimer and tetramer, respectively, for which no other oligomeric structures with a different catalytic activity have been reported. In comparison to the EntC-catalyzed forward reaction, these reactions are similarly affected in 30% Ficoll 70; the K_M values are significantly decreased while the k_{cat} values are slightly affected, resulting in a k_{cat}/K_M increase to a comparable magnitude (Table 1). The enhancement of LDH activity is similar to that reported for the same enzyme in concentrated Dextran solution.[9] Indeed, these results show that the rate enhancement effect of crowding is probably a general phenomenon.

Since K_M reduction was ascribable to an increase in chemical activity of the small molecule substrates in highly nonideal crowded solutions,[9] the activity coefficient of chorismate was first assessed for its contribution to the EntC rate enhancement. Distribution of

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Table 1. Enzyme Kinetic Parameters in 0% and 30% Ficoll 70

	0% Ficoll 70		30% Ficoll 70 (w/v)		
enzyme	K_M (μM)	k_{cat} (min^{-1})	K_M (μM)	k_{cat} (min^{-1})	relative k_{cat}/K_M
EntC	41.9 ± 3.0	290 ± 26	19.3 ± 0.5	236 ± 14	1.75 ± 0.16
EntB[a]	19.5 ± 1.8	325 ± 15	12.1 ± 1.8	365 ± 2	1.83 ± 0.29
MenF	3.76 ± 0.62	14.7 ± 1.6	1.50 ± 0.17	11.2 ± 0.3	2.02 ± 0.17
LDH[a]	524 ± 53	$(4.94 \pm 0.16) \times 10^4$	168 ± 21	$(3.49 \pm 0.49) \times 10^4$	2.19 ± 0.04

[a] EntB is a trimer and LDH is a tetramer in concentration calculation.

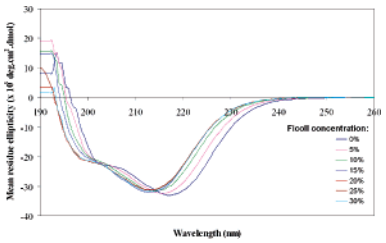

Figure 2. Circular dichroism spectra of EntC in Ficoll 70 solution.

chorismate was allowed to reach an osmotic equilibrium between a concentrated Ficoll solution and normal dilute buffer. The ratio of equilibrium chorismate concentration in the two solutions, which is reciprocal to the activity coefficient ratio, is between 1.0 and 1.2 in 0−15% Ficoll 70 (Figure S5). This small change in activity coefficient is consistent with the fact that crowding has minimal effect on the chemical activity of small molecules[2a,2b,6] and insufficient to account for the 2-fold K_M decrease that was observed in the same Ficoll concentration range (Figure 1). In addition, the rate enhancement is not caused by high viscosity of the crowded solutions because the EntC-catalyzed reaction is not diffusion-controlled and thus not susceptible to viscosity change, as pointed out earlier.[13] Even diffusion-controlled enzymic reactions involving small molecule substrates are not affected by viscosity increase caused by macromolecules.[13,14]

Besides the nonideality and high viscosity of the media, structure of the enzyme was also examined. A subtle conformational change was detected for EntC in Ficoll solutions by circular dichroism (CD). As shown in Figure 2, the CD spectrum does not change in shape but its negative minimum at 217 nm progressively shifts to 213 nm as Ficoll concentration is increased from 0 to 15%. At higher Ficoll concentrations, the spectrum remains unchanged. This spectral shift parallels the change of K_M, which is continuously decreased as Ficoll concentration is increased from 0 to 15% but remains steady at higher Ficoll concentrations (Figure 1). This strong correlation indicates the observed K_M decrease mainly results from the conformational change.

The conformational change is likely a result of domain repacking of EntC to reduce the excluded volume, under the entropy pressure imposed by macromolecular crowding. This is supported by the altered microenviroment of tryptophan (Trp) residues indicated by intensity decrease together with 1−2 nm red-shift of its fluorescence (Figure S6) under crowding conditions. In comparison, crowding-induced Trp fluorescence change for the allosteric ADP-glucose pyrophosphorylase is an intensity increase and a more pronounced blue-shift,[15] suggesting that crowding induces different structural change in different proteins. Moreover, enzymes under crowding conditions seem to generally undergo such structural change; conformational change also occurs in the other three enzymes (MenF, EntB, and LDH) in the crowded solutions, as evidenced by their CD spectrum change (Figure S7) and their Trp fluorescence red-shift and intensity decrease similar to EntC (Figure S6), although the pattern of spectral change differs. Despite the difference between individual enzymes, all the crowding-induced conformational change or repacking appears to directly affect the active sites, leading to an increase of their affinity for the substrates, as suggested by the congruent K_M decrease for all four enzymes in current study. This agrees well with the finding of the crowding-effected change in HIV-1 protease active site dynamics,[4] further suggesting that crowding may also alter the conformational flexibility and protein dynamics that are crucial in enzyme catalysis.[16]

In summary, we have demonstrated for the first time that macromolecular crowding increases the intrinsic activity of an enzyme in the absence of macromolecular association, through inducing conformational and possibly other structural changes in the enzyme. Although the rate enhancement is small, the congruent activity improvement for the four investigated enzymes suggests that it may occur in many other enzyme systems and, therefore, is not negligible in cellular biochemistry. Moreover, the crowding-induced structural change may also be a general phenomenon, considering the ubiquity of the highly crowded intracellular environment. These findings show that, in addition to imparting the well-known effects of promoting association and impeding diffusion of proteins, macromolecular crowding directly affects their structure and function.

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Supporting Information Available: Experimental procedures and Figures S0−S7. This material is available free of charge via the Internet at http://pubs.acs.org.

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