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Confinement and crowding effects on tryptophan synthase $\alpha_2\beta_2$ complex

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Abstract Biological molecules experience in vivo a highly crowded environment. The investigation of the functional properties of the tryptophan synthase $\alpha_2\beta_2$ complex either entrapped in wet nanoporous silica gels or in the presence of the crowding agents *dextran 70* and *ficoll 70* indicates that the rates of the conformational transitions associated to catalysis and regulation are reduced, and an open and less catalytically active conformation is stabilized.
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1. Introduction

It has been recently recognized both theoretically and experimentally that molecular crowding and confinement can produce profound effects on the dynamic and functional properties of biological macromolecules and affect processes like in vivo and in vitro folding, protein–ligand and protein–protein association, and enzyme catalysis [1–11]. A promising strategy to reproduce in vitro crowding and confinement effects is encapsulation of proteins in wet, nanoporous silica gels [12–23]. Following a reaction of hydrolysis and polycondensation of alkoxides, the entrapped molecules are individually caged in pores exhibiting a diameter of 20–50 Å, comparable to the protein size [24–26]. In silica gels, the spectroscopic and functional properties of the entrapped molecules are maintained, while excluded volume effects, the perturbation of water structure and non-covalent interactions with the gel matrix all contribute to alter protein stability, the kinetics of tertiary and quaternary conformational transitions [23,26–38] and the equilibrium distribution of conformations [32,38–40].

Tryptophan synthase (TS) is an $\alpha_2\beta_2$ complex containing a pyridoxal 5′-phosphate (PLP) bound to each β subunit [41] and catalyzing the last two steps of L-tryptophan biosynthetic pathway. The α subunit catalyzes the reversible cleavage of indole-3-glycerol-phosphate to produce D-glyceraldeyde-3-phosphate and indole, that is channeled via an hydrophobic tunnel to the β-site [42–44]. The β subunit catalyzes the PLP-dependent reaction between L-serine and indole to form L-tryptophan by a β-replacement mechanism. The β subunit also catalyzes a β-elimination reaction, leading to pyruvate and ammonia. Because the ratio of β-replacement vs. β-elimination activity is high, TS is classified as a β-replacement-specific enzyme [45]. An intricate set of allosteric interactions allows a fine tuning of α- and β-reactions [41,44,46–50] via regulatory signals that trigger the transition of α- and β-subunits between an *open*, catalytically inactive state, and a *closed*, catalytically active state [51–59].

Given the tight coupling between functional properties and conformational changes, TS represents an ideal system to investigate the influence of molecular crowding and confinement on enzyme catalytic activity and the transmission of regulatory signals. In this report, we have determined the catalytic activity and equilibrium distribution of intermediates for TS encapsulated in wet nanoporous silica gels and in the presence of the crowding agents *dextran 70* and *ficoll 70*, inert high molecular weight polysaccharides, the former being endowed by a more asymmetrical structure and a substantially higher viscosity than the latter [60].

2. Materials and methods

2.1. Reagents

Chemicals of the best available quality were obtained from Sigma, except L-serine (Fluka) and NADH and lactate dehydrogenase (Boehringer), and were used without further purification.

2.2. Enzyme

The TS $\alpha_2\beta_2$ complex was purified from *Escherichia coli* CB149 cells containing the plasmid pEBA10 carrying the *trpA* and *trpB* genes from *Salmonella typhimurium*. The growth of the bacterial strain and the purification were carried out according to Yang et al. [61].

2.3. Protein encapsulation

TS was encapsulated in tetramethylorthosilicate-derived wet silica gels, according to the procedure described by Ellerby et al. [12] with some modifications [29]. A sol derived from the acid-catalyzed hydrolysis of tetramethylorthosilicate, 50 mM potassium phosphate buffer, pH 8.0, and a solution containing 20 mg/ml TS in 25 mM bis-tris propane (BTP), pH 7.8, were mixed in a 0.5:0.5:1 ratio, at 4 °C. The mixture was quickly layered on a quartz slide, obtaining a thin gel film (~0.5 mm) within a few minutes. The gels were stored in 25 mM BTP, pH 7.0, 4 °C. Protein-doped gels were analyzed upon aging for at least 12 h.

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Abbreviations: TS, tryptophan synthase; PLP, pyridoxal 5′-phosphate; BTP, bis-tris propane; GP, α-D-glycerol-3-phosphate

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2.4. Activity assays

The catalytic activity of TS $\alpha_2\beta_2$ complex was assayed in solution in the absence and presence of different concentrations of *dextran 70* and *ficoll 70*, and in protein-doped silica gels, according to Ahmed et al. [62]. The reaction mixtures were incubated at 37 °C for 5 min. To avoid rate-limiting effects due to substrate diffusion, enzyme-doped gels were sonicated at low power to obtain a micron-size gel particle suspension. Optical inspection indicated that the average dimension of gel particles was about 4 μm. Sonication did not affect enzyme activity, as verified by applying the same protocol to the soluble enzyme. As in the case of tyrosine phenol-lyase and tryptophan indole lyase [38], it can be assumed that the negatively charged interior of the gel matrix does not affect the partitioning of zwitterionic substrates between the solution phase and the gel matrix [63]. To rule out the possibility that the measured activities are affected by enzyme molecules leached from the matrix, assay mixtures were filtered through a 0.22 μm filter. The filtrate did not exhibit any significant activity. Absorbance changes were monitored using a CARY219 spectrophotometer (Varian). 1-cm pathlength quartz cuvettes were used both for the soluble and the encapsulated enzyme. In the latter case, cuvettes were equipped with a magnetic stirrer. All assays were performed in triplicate. The determination of kinetic parameters was accomplished by fitting data with a non-linear regression to Michaelis–Menten equation using the software Sigma Plot 2000 (SPSS Science).

2.5. Spectrophotometric measurements

UV–vis absorption spectra were recorded with a CARY400 spectrophotometer (Varian) under different buffer conditions for the enzyme in solution, in the absence and presence of the crowding agents, and for the enzyme-doped silica gels. The absorption spectrum of a protein-free sample was subtracted to reduce the influence of light scattering originated from the non-perfect optical quality of the gel surface [38]. This subtraction may introduce some spectral distortion, especially at low wavelengths (300–350 nm).

3. Results

3.1. Dependence of TS catalytic activity on confinement and crowding agents

The catalytic activity of encapsulated TS was analyzed on a microsuspension of enzyme-doped silica gels, as previously carried out for tyrosine phenol-lyase and tryptophan indole-lyase [38]. Under this condition, the diffusion of substrates into the silica matrix does not limit catalytic rates. In fact, the diffusional limitations depend on several factors summarized in the following equation [62]:

$$d_c = \left[\frac{(K_M + [S_0]) \cdot D'}{k_{cat} \cdot [E]}\right]^{\frac{1}{2}} \quad (1)$$

where d_c is the fragment critical thickness over which rates are diffusion-controlled, K_M and k_{cat} are the catalytic parameters obtained in solution for TS from *S. typhimurium* (Table 1) [62], [E] is the enzyme concentration and $[S_0]$ the substrate concentration. D' is the diffusion coefficient of the substrate inside the gel calculated from the following equation:

$$\frac{D'}{D} = 1 - \frac{a^2}{r} \quad (2)$$

where a is the average molecular radius (~4 Å for molecules with molecular weight in the range 200–350), D ($\sim 6 \times 10^{-6}$ cm^2/s) is the diffusion coefficient in water for compounds in this range of molecular weight, and r (40–50 Å) is the average pore radius of the gel. The calculated values of d_c, reported in Table 1, are significantly higher than the average sample size of 4 μm, indicating that diffusion of substrates within gel microsuspensions cannot limit reaction rates. The β-replacement and β-elimination activity were assayed in solution and in silica gels using the natural substrate L-serine and the substrate analogue β-chloro-L-alanine (Table 2). The specific activity for the β-replacement reaction was found to be ninefold lower than in solution with L-serine, and threefold lower with β-chloro-L-alanine, indicating an *altered substrate specificity*. The specific activity for the β-elimination reaction of TS gel microsuspensions was 2.4-fold lower than in solution with L-serine and only 1.25-fold lower with β-chloro-L-alanine. Using L-serine as a substrate, the ratio of β-replacement vs. β-elimination activity in the gel is 11, a much lower value than that observed in solution, 44. This finding indicates an *altered reaction specificity*. The same conclusion can be drawn comparing the β-activities using β-chloro-alanine as a substrate. Furthermore, the presence of 50 mM glycerol-3-phosphate (GP), an α-subunit ligand acting as an allosteric effector of the β-subunit [41], causes small changes of the β-activity, compared to the fivefold decrease observed in solution (Table 2).

To understand whether the observed effects on the catalytic activity are due to alteration of substrate affinity or catalytic efficiency, we measured the dependence of catalytic rates on L-serine and indole concentrations in the gel and in solution. The substrate saturation curves were hyperbolic for all sets of conditions (data not shown), indicating that the enzymatic reactions follow Michaelis–Menten behavior. K_M values for the enzyme encapsulated in silica gels are only slightly higher than in solution (Table 3), whereas k_{cat} values are significantly reduced upon enzyme entrapment into a silica gel matrix (Table 3). For example, k_{cat} for L-serine in the β-replacement reaction is about 50-fold lower than in solution.

The effect of *dextran 70* and *ficoll 70* on the functional properties of the β-subunit of TS $\alpha_2\beta_2$ complex in solution was evaluated by determining the dependence of enzyme activity on the concentration of the crowding agents between 0 and 25% (w/v). The β-elimination reaction is scarcely affected by inert crowding agents, whereas a 20% decrease of β-replacement activity was observed.

Table 1
Catalytic parameters for TS from *S. typhimurium* in solution [62] and fragment critical thickness d_c for β-replacement and β-elimination reactions

	Indole (β-replacement)	L-Serine (β-elimination)
k_{cat} (s^{-1})	5.4	0.06
K_M (mM)	0.06	0.02
d_c (μm)	16.7	3117

Enzyme concentration was 70 μM, 0.2 mM indole and 100 mM L-serine.

Table 2
Catalytic activity of TS in solution and in the gel

	β-Replacement specific activity (μm/mg)			β-Elimination specific activity (μm/mg)	
	L-Serine		β-Cl alanine	L-Serine	β-Cl alanine
	−GP	+GP			
Solution	880 ± 10	185 ± 3	158 ± 9	20 ± 1	25 ± 1
Gel	95 ± 5	73 ± 1	53 ± 1	8.4 ± 0.1	20 ± 1

The β-replacement reaction with L-serine was assayed in the absence and presence of 50 mM GP.

Table 3
Kinetic parameters for β-replacement and β-elimination reactions catalyzed by TS in solution and in the gel

		Solution			Gel		
		K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})
β-Replacement	Indole	0.094 ± 0.006	10.1 ± 2.4	107.4	0.15 ± 0.03	3.3 ± 0.3	22.0
	L-Serine	0.98 ± 0.03	8.2 ± 0.1	8.4	1.17 ± 0.16	0.16 ± 0.05	0.14
β-Elimination	L-Serine	1.16 ± 0.19	0.26 ± 0.01	0.22	1.41 ± 0.40	0.15 ± 0.01	0.11

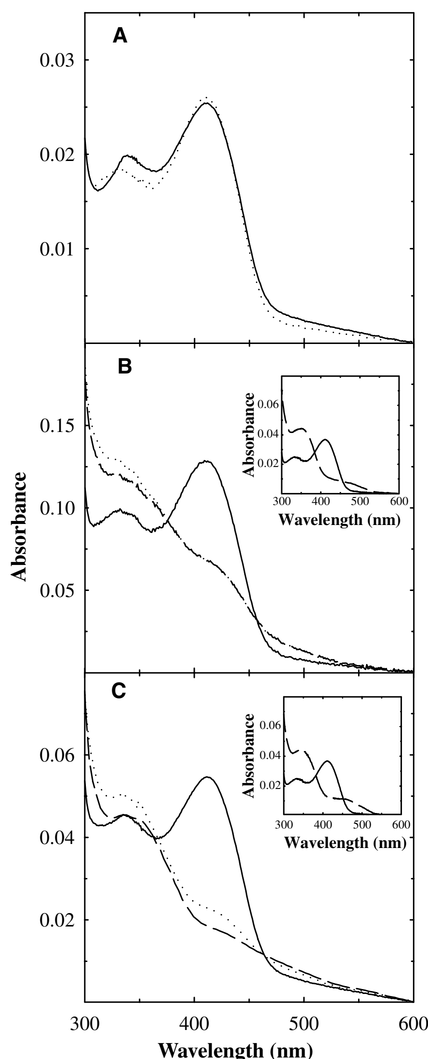

Fig. 1. Comparison of absorption spectra of TS, in the absence and presence of L-serine, in solution and in silica gels. (A) Absorption spectrum of the internal aldimine of TS-doped gels (solid line) and soluble TS (dashed line) in a solution containing 25 mM BTP, pH 7.0, 25 °C. Spectra were normalized for the absorption intensity at 280 nm. (B) Absorption spectra of TS-doped gels in the absence (solid line) and in the presence of 100 mM L-serine, 25 mM BTP, pH 7.0, 25 °C, before (dotted line) and after subtraction (dashed line) of the spectrum of pyruvate formed due to the β-elimination reaction. Inset: Absorption spectra of soluble TS in the absence (solid line) and in the presence (dashed line) of 100 mM L-serine, 25 mM BTP, pH 7.0, 25 °C. (C) Absorption spectra of TS-doped gels in the absence (solid line) and in the presence of 100 mM L-serine, 100 mM CsCl, 25 mM BTP, pH 7.0, 25 °C, before (dotted line) and after subtraction (dashed line) of the spectrum of pyruvate formed due to the β-elimination reaction. Inset: Absorption spectra of soluble TS in the absence (solid line) and in the presence (dashed line) of 100 mM L-serine, 100 mM CsCl, 25 mM BTP, pH 7.0, 25 °C.

3.2. Absorption spectra of TS catalytic intermediates

The absorption spectrum of TS-doped silica gels exhibits two major bands centered at 412 and 340 nm, attributed to the polar ketoenamine and the apolar enolimine tautomers of the internal aldimine, respectively (Fig. 1A) [64]. The ratio of band intensity is lower (~1.2) in the gel than in solution (~1.7), suggesting that the enolimine tautomer is slightly more populated in the entrapped form of the enzyme. A shift of the tautomeric equilibrium was also observed for tyrosine phenol lyase and tryptophan indole lyase in silica gels [38].

In the absence of indole, the reaction of L-serine with the β-subunit of the TS $\alpha_2\beta_2$ complex leads to the formation of a distribution of the catalytic intermediates external aldimine and α-aminoacrylate Schiff base, absorbing at 420 and 350 nm, respectively [65]. This distribution is strongly affected by pH, monovalent cations, α-subunit ligands and temperature [54,66–68]. We observed that in the gel the steady-state distribution of intermediates is reached on a much longer time scale (about 2 h) than in solution (within the mixing time). Both a reduction in protein flexibility, and a mass transport limitation might explain this behavior. In fact, the gel thickness was about 0.5 mm, thus likely slowing down the equilibration of enzymes sites with the external medium. However, gel thickness cannot be the only cause of the reduction in equilibration rates. In fact, it has been demonstrated that fluorescein concentration equilibrates in seconds throughout gels of comparable thickness (Cannone F., personal communication). Absorption spectra of TS-doped silica gels with L-serine in the absence and presence of cesium ions, recorded after 180 min, are shown in Fig. 1B and C. Absorption spectra were corrected for the contribution of pyruvate, produced by the β-elimination reaction and evaluated by the lactate dehydrogenase assay, as previously reported [38]. The comparison between TS absorption spectra in the gel and in solution (Fig. 1B and C, inset) indicates that, for the entrapped enzyme, the equilibrium distribution of catalytic intermediates is shifted in favor of the external aldimine.

Absorption spectra of TS in solution, in the absence and presence of L-serine, with increasing concentration of *dextran 70* and *ficoll 70* were also collected. The equilibrium distribution of the ketoenamine and enolimine tautomers of the internal aldimine was not significantly altered (data not shown). Similarly, the equilibrium distribution of the external aldimine and α-aminoacrylate was not affected by increasing concentrations of the crowding agents (data not shown).

4. Discussion

Encapsulation in silica gels affects the catalytic activity of several enzymes, inducing a 3–4000-fold decrease of k_{cat} and

an increase of K_M [22,23,69]. Frequently, this reduced activity is the consequence of catalytic reactions limited by substrate diffusion within thick gels. However, even in studies where gel thickness was selected to avoid diffusion limitations, a reduction of enzyme activity was detected [38], indicating that encapsulation may introduce constraints to protein flexibility or stabilize inactive enzyme conformations. A remarkable exception is represented by lipase encapsulated in hydrophobic silica gels where activity increases ~1000-fold [70] due to gel-induced stabilization of the open conformation of the enzyme allowing free access of substrates to the active site. Furthermore, investigations of entrapped PLP-dependent tyrosine phenol lyase and tryptophan indole lyase suggested that the observed decrease in catalytic activity was due to both an altered conformational equilibrium between enolimine and ketoenamine tautomers and kinetic restrictions to catalytically relevant conformational changes [38]. In the case of TS the encapsulation appears to act at different levels. The comparison of specific activities for β-replacement and β-elimination reactions in solution and in silica gels reveals an alteration of both substrate and reaction specificity. For encapsulated TS, in the β-replacement reaction β-chloro-L-alanine becomes a substrate almost as good as L-serine, whereas in solution there is a sixfold difference in specific activity. The determination of steady-state catalytic parameters provides evidence that changes in the overall catalytic efficiency (k_{cat}/K_M) for each reaction mostly depend on the reduction of k_{cat} values, whereas K_M values are only slightly affected. Moreover, the ratio of activity between the β-replacement and β-elimination reaction with either L-serine or β-chloro-L-alanine is fourfold lower in silica gels than in solution, suggesting that encapsulation has altered the distribution of enzyme conformations increasing the population of the conformational state that preferentially catalyzes the β-elimination over the β-replacement. This state is likely to correspond to the open conformation. In fact, in the open form, the Schiff base of the aminoacrylate preferentially undergoes a β-elimination with respect to a β-replacement reaction due to a higher solvent accessibility [71]. In solution, the transition to the closed form of the enzyme provides the rationale for favoring the productive β-replacement reaction. Moreover, the reduced effects observed with β-chloro-L-alanine might be explained by the less stringent geometric complementarity between the substrate and the active site. The presence of a very good leaving group, the chloride ion, might make the substrate less sensitive to active site conformations. Overall, the results suggest that encapsulation in silica gel alters the equilibrium distribution between *open* and *closed* forms of TS β-subunit, favoring the *open* form. Spectroscopic results support this interpretation. In fact, in the presence of L-serine TS-doped silica gels slowly react forming the catalytic intermediates external aldimine and α-aminoacrylate, with the external aldimine more populated than in solution. Solution studies demonstrated that the open and closed structures are predominantly associated to the external aldimine and α-aminoacrylate intermediates, respectively [54]. Interestingly, microspectrophotometric studies carried out on TS crystals in the presence of L-serine indicated that the equilibrium distribution was also shifted in favor of the external aldimine species with respect to solution [68]. Furthermore, it appears that allosteric signals transmitted from the α- to the β-active site and mediated by selective stabilization of the closed conformations are damped by encapsulation, as judged by the negligible influence of GP on β-replacement activity in comparison with solution. The emerging picture is that encapsulation in silica gel not only slows down conformational transitions but also stabilizes defined protein conformations [38].

In order to resolve, at least partially, how much of the observed effects on TS function and dynamics is related to specific interactions with the gel matrix, confinement in the finite space of the gel pores and altered solvent structure, we have investigated the influence on catalytic and spectroscopic properties of the crowding agents *dextran 70* and *ficoll 70* [2,3]. These compounds are known as ideal crowding agents because they interact with macromolecules only via steric repulsion. According to the excluded volume theory, *dextran 70* and *ficoll 70* should selectively stabilize more compact protein conformations and affect the equilibrium and kinetics of any macromolecular reaction in which there is a significant difference between the volume excluded to reactants and the volume excluded to products. We have observed that these crowding agents do not significantly affect the spectroscopic and functional properties of TS, thus suggesting only small volume differences between conformational states. This result indicates that, at least in the case of silica gel-encapsulated TS, the altered equilibrium distribution of tertiary conformations can not be totally ascribed to caging and viscosity effects, but might have a strong contribution from protein-matrix interactions.

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