Monovalent Cations Affect Dynamic and Functional Properties of the Tryptophan Synthase $\alpha_2\beta_2$ Complex†

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Received December 22, 1994; Revised Manuscript Received April 18, 1995⊗

ABSTRACT: Monovalent cations affect both conformational and catalytic properties of the tryptophan synthase $\alpha_2\beta_2$ complex from *Salmonella typhimurium*. Their influence on the dynamic properties of the enzyme was probed by monitoring the phosphorescence decay of the unique Trp-177β, a residue located near the β-active site, at the interface between α- and β-subunits. In the presence of either Li^+, Na^+, Cs^+, or NH_4^+, the phosphorescence decay is biphasic and the average lifetime increases indicating a decrease in the flexibility of the N-terminal domain of the β-subunit. Since amplitudes but not lifetimes are affected, cations appear to shift the equilibrium between preexisting enzyme conformations. The effect on the reaction between indole and L-serine was studied by steady state kinetic methods at room temperature. We found that cations: (i) bind to the L-serine−enzyme derivatives with an apparent dissociation constant, measured as the concentration of cation corresponding to one-half of the maximal activity, that is in the millimolar range and decreases with ion size; (ii) increase k_{cat} with the order of efficacy $Cs^+ > K^+ > Li^+ > Na^+$; (iii) decrease K_M for indole, Na^+ being the most effective and causing a 30-fold decrease; and (iv) cause an increase of the k_{cat}/K_M ratio by 20−40-fold. The influence on the equilibrium distribution between the external aldimine and the α-aminoacrylate, intermediates in the reaction of L-serine with the β-subunits of the enzyme, was found to be cation-specific. In the absence of cations, at pH 7.9, the predominant species is the α-aminoacrylate absorbing at 350 nm; Cs^+, Rb^+, and Li^+ cause the formation of a new absorption at about 470 nm, tentatively assigned to a tautomer of the α-aminoacrylate, whereas Na^+ and K^+ stabilize the external aldimine absorbing at 422 nm. By single-crystal polarized absorption microspectrophotometry, we verified that the cation-specific effects on the equilibrium distribution of β-subunit intermediates are maintained in the crystalline state. These studies help to define the experimental conditions suitable for X-ray crystallographic studies aiming to identify the cation-binding sites and their mode of action.

Since the discovery of the K^+-activating effect on pyruvate kinase (Boyer et al., 1942), many enzymes have turned out to be specifically regulated by monovalent cations (Suelter, 1970). In contrast to well-defined functions assigned to divalent cations in enzyme catalysis and to detailed structural information on their mode of interaction with proteins, the role of monovalent cations in catalysis and/or regulation is still obscure. Up to now, the three-dimensional structure of only three monovalent cation-dependent enzymes has been determined, the PLP[1]-dependent dialkylglycine decarboxylase (Toney et al., 1993; Hohenester et al., 1994), pyruvate kinase (Larsen et al., 1994), and RNase T1 (Koepke et al., 1989), while the structural analysis of two other PLP-dependent enzymes, tyrosine phenol-lyase (Antson et al., 1994) and tryptophanase (Isupov et al., 1994), is in progress. Moreover, the structures of a few proteins that bind, but do not require for activity, monovalent cations have been reported (Gursky et al., 1992; Badger et al., 1994a,b; Pantoliano et al., 1988; Gros et al., 1989).

In his review of enzymes activated by monovalent cations, Suelter (1970) pointed out that most of the catalyzed reactions proceed via structurally similar key intermediates. In particular, the observation that several PLP-dependent enzymes catalyzing β-elimination reactions are regulated by monovalent cations led to the hypothesis that these cations specifically interact with the common coenzyme-bound intermediate α-aminoacrylate.

Bacterial tryptophan synthase is a PLP−$\alpha_2\beta_2$ complex that catalyzes the two final steps in the biosynthesis of L-tryptophan. First, α-subunits cleave indole-3-glycerol phosphate to form indole and glyceraldehyde-3-phosphate (α-reaction). Then, indole is channeled to the β-active site and is condensed with the PLP-bound α-aminoacrylate Schiff base, resulting from the β-elimination reaction of L-serine (β-reaction) (Yanofsky & Crawford, 1972; Miles, 1979, 1991).

The enzyme is allosterically regulated, i.e., ligands of one subunit alter the functional properties of the other (Kirschner et al., 1975, 1991; Lane & Kirschner, 1981, 1983a,b, 1991; Kawasaki et al., 1987; Mozzarelli et al., 1989, 1991; Dunn et al., 1987, 1990, 1991; Houben & Dunn, 1990; Anderson et al., 1991; Brzovic′ et al., 1992a−c). In particular, the equilibrium distribution between the α-aminoacrylate and the external aldimine species exhibits a pH dependence which is affected by the α-subunit ligand glycerol-3-phosphate as well as by temperature (Mozzarelli et al., 1989, 1991; Rossi et al., 1992; Peracchi et al., unpublished observations). By

† This work was supported by funds from the Italian Ministry of Universities and Research in Science and Technology (MURST) and the Italian National Research Council (CNR) Target Project on Biotechnology and Bioinstrumentation.

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⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1995.

[1] Abbreviations: PLP, pyridoxal-5′-phosphate; GP, D,L-α-glycerol-3-phosphate.

0006-2960/95/0434-9459$09.00/0

luminescence measurements, it was also shown that the α-subunit ligand glycerol-3-phosphate stabilizes a more compact conformation of the enzyme (Strambini et al., 1992b). During these investigations we found that monovalent cations strongly affect the equilibrium distribution of catalytic intermediates at the β-site. Previous experiments had shown that the $\alpha_2\beta_2$ complex from *Escherichia coli* exhibits a significant decrease of synthase activity in the presence of 3 N ammonium citrate (Hatanaka et al., 1962), while the *Bacillus subtilis* enzyme shows an increase of activity in the presence of 0.2−0.8 M potassium chloride or sodium chloride (Schwartz & Bonner, 1964). Surprisingly, no further studies were carried out to characterize the effects of monovalent cations on structural and functional properties of the $\alpha_2\beta_2$ complex. However, it is well known that monovalent cations increase the rate of L-tryptophan synthesis, catalyzed by the isolated β_2 dimer, with the order of activation $NH_4^+ > Li^+ > K^+ > Na^+$. In particular, it was found that NH_4^+ decreases 10 times the K_M for L-serine, while it does not affect the K_M for indole (Hatanaka et al., 1962). Monovalent cations also stimulate the L-serine deamination, a side reaction catalyzed by the β-subunit, that forms pyruvate and ammonium ion (Crawford & Ito, 1964). Moreover, the rate of the α-reaction, either catalyzed by the isolated α-subunit (Hatanaka et al., 1962) or by the $\alpha_2\beta_2$ complex (Dunn et al., 1994), is decreased by monovalent cations. These findings suggest the presence of a cation-binding site on both subunits. The three-dimensional structure of the $\alpha_2\beta_2$ complex from *Salmonella typhimurium*, determined either in the absence or in the presence of an α-subunit ligand, did not provide immediate evidence for the presence of a monovalent cation-binding site (Hyde et al., 1988).

In the present work we characterize the effects of monovalent cations on L-tryptophan synthesis catalyzed by the $\alpha_2\beta_2$ complex from *S. typhimurium* and on the distribution of catalytic intermediates formed in the reaction of the β-subunit of the enzyme with L-serine. Since the enzyme in the crystalline state was shown to be catalytically competent (Ahmed et al., 1987; Mozzarelli et al., 1989), we have extended these studies to the crystalline enzyme in order to verify whether the cation effects are maintained in this physical state and to eventually define experimental conditions suitable for the identification of cation-binding sites. Furthermore, to address the question of whether cation binding affects the conformational properties of the enzyme, we have monitored either in the absence or in the presence of cations the phosphorescence decay of the unique Trp-177β, a probe suitably located at the subunit interface (Strambini et al., 1992a,b). A preliminary report of part of this investigation has been presented at the 9th vitamin B6 meeting (Peracchi et al., 1994).

MATERIALS AND METHODS

The tryptophan synthase $\alpha_2\beta_2$ complex from *S. typhimurium* was purified from the *E. coli* strain containing the plasmid encoding the *S. typhimurium* genes, according to Kawasaki et al. (1987). Crystals of the enzyme were grown from poly(ethylene glycol) solutions as previously described (Ahmed et al., 1987; Mozzarelli et al., 1989). Plasticware was preferred to glassware. Salts used in this study contained chloride as the counteranion. In a few cases, fluoride or acetate salts were used and found not to change the results. Na^+ and K^+ concentration in reaction buffers was checked by atomic absorption spectroscopy and found to be less than 17 and 5 μM, respectively. All reagents were of the best commercially available quality and used without further purification. Experiments were carried out in 25 mM bis-Tris-propane buffer. Adjustment of pH in this buffer was performed by addition of hydrochloric acid. pH measurements were carried out using a pHM 83 radiometer equipped with a U402-M3 Ingold microelectrode.

Before measurements, the enzyme was extensively dialyzed three times against a 100-fold excess solution containing 25 mM bis-Tris-propane, 1 mM dithiothreitol, and 4 μM PLP. The dialyzing buffer for the enzyme used in phosphorescence measurements did not contain dithiothreitol. The reaction of the $\alpha_2\beta_2$ complex with L-serine and indole in the presence and absence of cations was monitored at 290 nm in a solution containing 10 μM PLP and 25 mM bis-Tris-propane, pH 7.9, at 25 °C. Enzyme activity was expressed in Yanofsky units. One unit corresponds to a ΔA at 290 nm of 0.185 in 20 min, which is equal to the conversion of 0.1 μmol of substrate to product.

Absorption spectra of a solution containing the enzyme, 50 mM L-serine, 25 mM bis-Tris-propane, pH 7.9, and different cations were recorded at both 10 and 25 °C. The dissociation constant for Na^+ and K^+ was determined by monitoring the fluorescence of the external aldimine (λ_{em} = 500 nm; λ_{ex} = 420 nm), the only highly fluorescent catalytic species (Goldberg et al., 1968).

Polarized absorption spectra of the $\alpha_2\beta_2$ complex were recorded on single crystals suspended in a medium containing 20% (w/v) PEG, M_r 8000, 4 μM PLP, and 25 mM bis-Tris-propane, pH 7.9, either in the absence or in the presence of monovalent cations, at 20 °C. While 2 mM spermine was present in the crystal mother liquor, it was omitted in the crystal-suspending medium. Polarized absorption measurements on monoclinic crystals of tryptophan synthase were carried out by a Zeiss UV MPM03 microspectrophotometer, as previously described (Mozzarelli et al., 1989).

Phosphorescence measurements were carried out at pH 7.8, 0 °C, as previously described (Strambini et al., 1992a,b). Trp-177β was excited at 298 nm, and the phosphorescence emission was monitored at 420 nm.

Steady state kinetic and titration data were analyzed by a nonlinear least squares fitting to the appropriate equation with an algorithm available on SigmaPlot software (Jandel Scientific).

RESULTS

Steady State Kinetics of L-Tryptophan Formation Catalyzed by the $\alpha_2\beta_2$ Complex in the Presence and Absence of Cations. The rate of L-tryptophan synthesis, catalyzed by the tryptophan synthase $\alpha_2\beta_2$ complex in the presence of L-serine and indole, is increased by monovalent cations (Figure 1). The order of activation is $NH_4^+ > Cs^+ > Rb^+ > Li^+ > K^+ > Na^+$ with NH_4^+ increasing the reaction rate by about 5 times. The observed effect is not due to an increase of ionic strength, since the bulky monovalent cations triethylammonium and tetramethylammonium or the divalent cations Mg^{2+} and Ca^{2+} do not affect the reaction. Furthermore, it is not associated with a shift of the pH dependence of the activity induced by monovalent cations (Mozzarelli, unpublished results). Furthermore, a 10-fold increase in PLP concentration present in the activity assay or a 2-fold excess of α-subunit concentration does not change the observed rate of reaction, indicating that the low activity measured in the

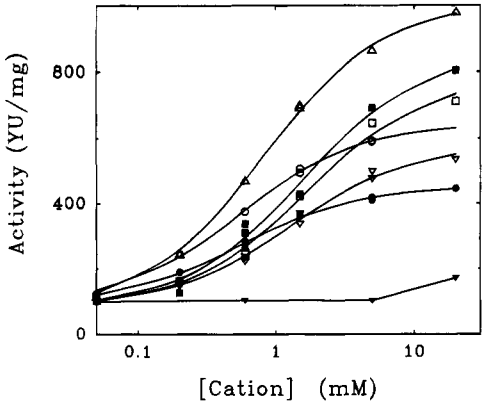

FIGURE 1: Effects of cation concentration on the rate of L-tryptophan synthesis. Enzymatic activity was assayed in a solution containing 50 mM L-serine, 0.2 mM indole, 10 μM PLP, and 25 mM bis-Tris-propane, pH 7.9, in the presence of increasing concentration of tetramethylammonium$^+$ (▼), Li^+ (○), Na^+ (●), K^+ (▽), Rb^+ (□), Cs^+ (■), and NH_4^+ (△), at 25 °C. The curve through data points represents the least squares fitting to the equation of a binding isotherm. The calculated $K_{activation}$ values are reported in Table 1.

Table 1: Activation Constants and Steady State Kinetic Parameters for L-Tryptophan Synthesis in the Absence and Presence of Monovalent Cations, at 25 °C

cation	ionic radius (Å)	$E_{hydration}$ (kcal/mol)	$K_{activation}$ (mM)	K_M (indole) (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1} × 10^3)
				590 ± 65	2.46 ± 0.13	4.17
Li^+	0.60	−98	0.55 ± 0.04	44 ± 8	4.27 ± 0.19	97.0
Na^+	0.95	−72	0.56 ± 0.04	18 ± 3	3.41 ± 0.11	189
K^+	1.33	−55	1.31 ± 0.16	95 ± 23	4.85 ± 0.32	51.1
Rb^+	1.48	−51	1.70 ± 0.25			
Cs^+	1.69	−47	1.60 ± 0.27	67 ± 15	5.44 ± 0.25	81.2
NH_4^+	1.49		0.81 ± 0.06			

absence of monovalent cations is not due to subsaturation of the coenzyme sites or to subunit dissociation. To exclude inhibitory effects of bis-Tris-propane on the reaction, activity assays were carried out in 25 mM *N*-ethylmorpholine or 25 mM triethanolamine. The specific activity was found to be buffer-independent (data not shown).

The dependence of the specific activity on monovalent cation concentration was fitted to a binding isotherm (Figure 1). The apparent activation constant for each monovalent cation (i.e., the cation concentration required to obtain one-half of the maximal activation) was found to be in the millimolar range and to increase as a function of the cation radius (Table 1). At cation concentrations higher than 20 mM, we observed an inhibitory effect of sodium, calcium, and magnesium ions and a much smaller stimulatory effect of lithium, cesium, and ammonium ions, possibly suggesting the presence of a secondary cation-binding site (data not shown).

Cations can affect the rate of L-tryptophan synthesis by altering k_{cat} and/or K_M for L-serine or indole or both. We first determined the affinity of L-serine for the enzyme in the absence and presence of 20 mM sodium or 20 mM cesium ions by recording absorption spectra as a function of increasing concentrations of L-serine, at pH 7.9, 10 °C (data not shown). The calculated dissociation constant is 91 μM in the absence of monovalent cations and 61 or 22 μM in the presence of Na^+ or Cs^+, respectively. Therefore, cations have a relatively small effect on L-serine affinity, and moreover, enzyme sites are fully saturated at the L-serine

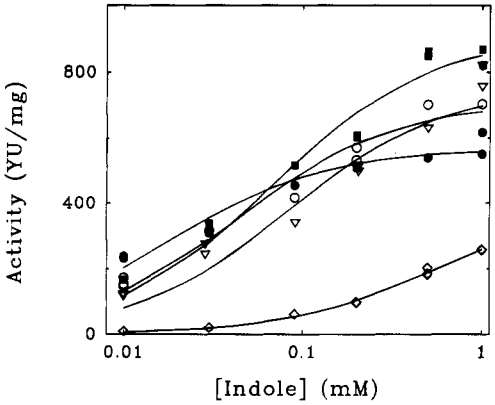

FIGURE 2: Dependence of the rate of L-tryptophan synthesis on indole concentration in the presence and absence of monovalent cations. The rate of reaction was determined in an assay solution that contained 50 mM L-serine in the absence (◇) and presence of Li^+ (○), Na^+ (●), K^+ (▽), and Cs^+ (■) at indole concentrations varying between 0.01 and 1 mM, at 25 °C. The catalytic parameters (reported in Table 1) were obtained by fitting the data to the Michaelis−Menten equation.

concentration used in the assay solution (50 mM) both in the absence and in the presence of monovalent cations.

Activity assays were then carried out in the presence of a saturating concentration (20 mM) of cation and increasing concentrations of indole, at 25 °C (Figure 2). The apparent K_M for indole and k_{cat}, calculated by fitting the data to the Michaelis−Menten equation, are reported in Table 1. Monovalent cations affect both the K_M and the k_{cat}. The resulting k_{cat}/K_M values in the presence of monovalent cations (Table 1) are 20−40 times higher than in the absence of cations. Interestingly, sodium ion is the most effective and cesium the least effective cation.

Effects of Monovalent Cations on Conformational Properties of the $\alpha_2\beta_2$ Complex. The phosphorescence decay of Trp-177β was monitored either in the absence or in the presence of monovalent cations. In the absence of cations, the decay is biexponential, with a predominant fast phase (Table 2). In the presence of either Li^+, Na^+, Cs^+, or NH_4^+, the phosphorescence decay is still biphasic with a small difference in lifetimes but a significantly increased amplitude of the slow phase (Table 2). As a consequence, the average lifetime of the decay ($\tau_{av} = \tau_1\alpha_1 + \tau_2\alpha_2$) is longer in the presence than in the absence of monovalent cations, thus indicating that the protein becomes less flexible when cations bind to the protein.

Reaction of the $\alpha_2\beta_2$ Complex with L-Serine in the Presence of Monovalent Cations in Solution. The reaction of L-serine with the $\alpha_2\beta_2$ complex leads to the formation of an equilibrium distribution of intermediates (Miles, 1979; Drewe & Dunn, 1985). This equilibrium is affected by pH, α-subunit ligands, and temperature (Mozzarelli et al., 1989, 1991). In the absence of cations, at pH 7.9, 25 °C (Figure 3a), the spectrum is characterized by a predominant band at 350 nm and a pronounced shoulder at 422 nm. The 350 nm absorption band has been attributed to the α-aminoacrylate Schiff base (Miles, 1979, 1991; Drewe & Dunn, 1985), whereas the 422 nm absorbing species is the L-serine external aldimine (Goldberg et al., 1968). This distribution is affected by the presence of monovalent cations (Figure 3a). Binding of Na^+ or K^+ favors the accumulation of the external aldimine, whereas binding of Li^+, Rb^+, and Cs^+ causes the formation of a broad band at 470 nm and a blue shift of the 350 nm band. The stabilization of the external aldimine by

Table 2: Effect of Monovalent Cations on the Phosphorescence Decay of Tryptophan-177β[a]

cation	M^+ (mM)	τ_1 (ms)	τ_2 (ms)	α_1	α_2	τ_{av} (ms)[b]
		22.9 ± 1.6	61.4 ± 13.5	0.88 ± 0.06	0.12 ± 0.06	27.5 ± 4.3
Li^+	10	24.0 ± 2.4	50.1 ± 5.0	0.60 ± 0.19	0.40 ± 0.19	34.5 ± 10.8
Na^+ [c]	20	28.2	48.5	0.46	0.54	39.1
Na^+	280	29.3 ± 3.3	56.1 ± 4.2	0.61 ± 0.15	0.39 ± 0.15	39.8 ± 9.8
Cs^+	10	22.0 ± 3.0	51.3 ± 3.8	0.47 ± 0.06	0.53 ± 0.06	37.5 ± 4.2
NH_4^+	10	20.5 ± 0.5	45.0 ± 1.0	0.46 ± 0.05	0.54 ± 0.05	34.5 ± 2.5

[a] Time courses were followed at 420 nm upon excitation at 298 nm, at pH 78, 0 °C. [b] $\tau_{av} = \tau_1\alpha_1 + \tau_2\alpha_2$. [c] Data from Strambini et al. (1992a).

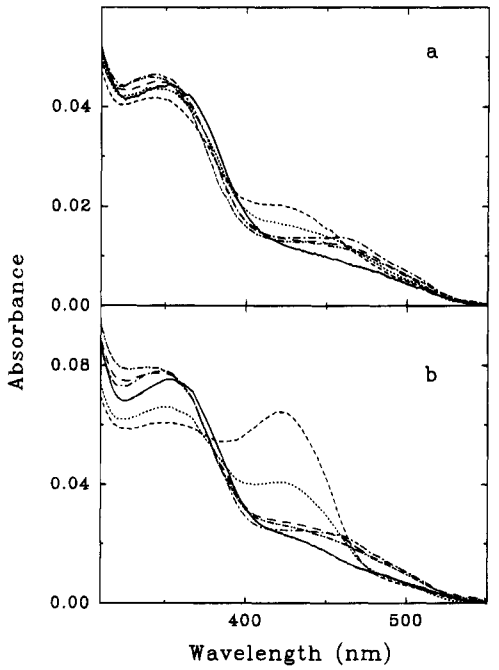

FIGURE 3: Absorption spectra of L-serine−$\alpha_2\beta_2$ derivatives in the presence and absence of monovalent cations. The $\alpha_2\beta_2$ complex (0.6 mg/mL, panel a; 0.9 mg/mL, panel b) was incubated in a solution containing 50 mM L-serine, 25 mM bis-Tris-propane, and 1 mM EDTA, pH 7.9, at either 25 °C (panel a) or 10 °C (panel b). Absorption spectra of the reaction mixture were recorded in the absence of added cations (—) and in the presence of 20 mM Li^+ (— •• —), Na^+ (- - -), K^+ (•••), Rb^+ (— — —), and Cs^+ (— • — • —).

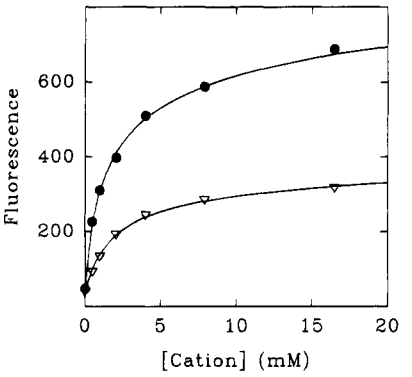

FIGURE 4: Binding of Na^+ and K^+ to L-serine−$\alpha_2\beta_2$ derivatives. Fluorescence at 500 nm (λ_{ex} = 420 nm) was recorded for a solution containing the enzyme (0.1 mg/mL), 50 mM L-serine, 25 mM bis-Tris-propane, and increasing concentrations of Na^+ (●) and K^+ (▽), pH 7.9, at 10 °C. The solid lines through the data points are the best least squares fit to two independent binding isotherms.

Na^+ and K^+ is significantly more pronounced when spectra of the reaction mixture are recorded at 10 °C (Figure 3b). Since formation of the external aldimine is accompanied by an increase of fluorescence at 500 nm (Goldberg et al., 1968), the binding affinity of Na^+ or K^+ to the L-serine−enzyme complexes was investigated at pH 7.9, 10 °C, by measuring the fluorescence change. The dependence of fluorescence on cation concentration is biphasic (Figure 4), suggesting the presence of two cation-binding sites. The dissociation constants, calculated by fitting the data to two independent binding isotherms, are 1.0 and 18.7 mM for sodium and 1.8 and 189 mM for potassium. The dissociation constants of the tight binding site for both Na^+ and K^+ are similar to those found under steady state conditions (Table 1).

Reaction of the Crystalline $\alpha_2\beta_2$ Complex with L-Serine in the Presence of Monovalent Cations. Single-crystal polarized absorption spectra of the L-serine−enzyme complexes, recorded in the absence of cations, at pH 7.9, 20 °C (Figure 5), exhibit a band at 350 and 422 nm, indicating that both the α-aminoacrylate and the external aldimine are formed in the crystalline enzyme (Mozzarelli et al., 1989). The equilibrium distribution is shifted in favor of the latter species with respect to solution (Figure 5a). The presence of Na^+ in the crystal-suspending medium increases the amount of the external aldimine, as observed in solution. However, the effect is much stronger than in solution, as evidenced by the complete disappearance of the 350 nm band and the appearance of a well-shaped band centered at 422 nm. On the other hand, the presence of Cs^+ causes an increase of the absorption in the 470−480 nm region and a blue shift of the 350 nm band (Figure 5b), in analogy with that observed in solution. The apparent dissociation constant of Na^+ for the crystalline L-serine−enzyme derivatives is 15 mM (Figure 5a, inset), and that of Cs^+ is 1.8 mM (Figure 5b, inset).

DISCUSSION

In the present paper we have demonstrated that the functional properties of the tryptophan synthase $\alpha_2\beta_2$ complex are strongly dependent on binding of monovalent cations, and by monitoring different physicochemical parameters affected by cations, we are able to provide a possible linkage between dynamic properties of the enzyme and catalytic efficiency. Furthermore, by showing that the functional effects of cations are conserved in the crystalline state, we have set the basis for a detailed crystallographic investigation of the phenomenon.

Ion-Binding Site and Ion Selectivity. Binding of ions to tryptophan synthase has been followed by their ability to elicit the β-reaction. We have found that this effect is specific of monovalent cations. At monovalent cation concentrations lower than 20 mM, the activation is well described by a single, noncooperative binding process, compatible with a minimal model in which only one high-affinity site exists per $\alpha\beta$ dimer. Although no attempt was made to locate such a binding site, the well-known existence of a similar activation in the isolated β_2 dimer (Hatanaka et al., 1962; Crawford & Ito, 1964) strongly suggests that cations specifically bind to the β-subunit. This assumption has been recently confirmed by the X-ray analysis of enzyme

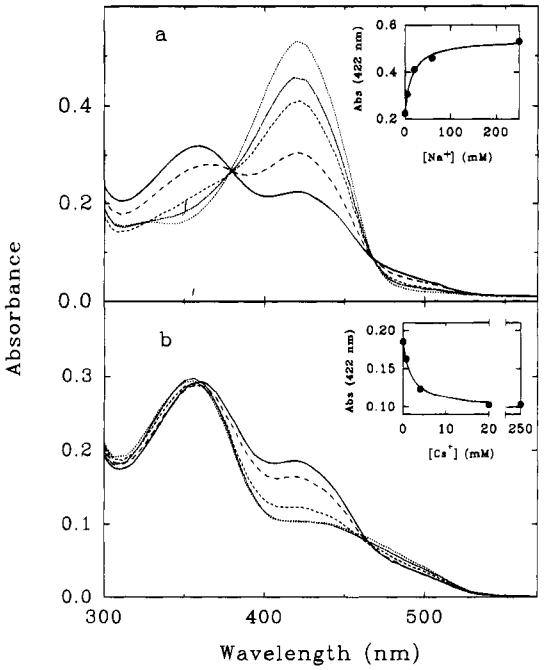

FIGURE 5: Effects of cations on the distribution of intermediates formed in the reaction between L-serine and the $\alpha_2\beta_2$ complex in the crystalline state. Polarized absorption spectra of monoclinic tryptophan synthase crystals were recorded with the electric vector of the incident light parallel to the x optical axis of the (210) face, where the transition dipole moments of PLP mainly project (Mozzarelli et al., 1989). The crystal was suspended in a solution containing 25 mM bis-Tris-propane, 20% PEG (8000 M_r), 50 mM L-serine, pH 7.9, at 20 °C, and increasing concentration of Na^+ (panel a, 0 (—), 0.5 mM (— — —), 20 mM (— — —), 60 mM (- - -), and 250 mM (•••)) and Cs^+ (panel b, 0 (—), 0.8 mM (— — —), 4 mM (— — —), 20 mM (- - -), and 250 mM (•••)). Inset: absorbance at 422 nm as a function of cation concentration was least squares fitted to a binding isotherm. The calculated apparent dissociation constant is 15 mM for Na^+ and 1.8 mM for Cs^+.

crystals, soaked in the presence of different cations (Na^+, K^+, and Cs^+) (Drs. David Davies, Kevin Parris, and Sankee Rhee, personal communication), that indicates that the cation-binding site is localized near the β-active site.

The activation constants calculated for the various ions (Table 1) can be considered overall dissociation constants for the substrate—enzyme complexes and provide valuable information on site specificity and energetics of binding. The low selectivity of cation binding by tryptophan synthase is striking and unexpected. In fact, most of the proteins that bind monovalent cations are very selective toward K^+ due to the balance of entropic and enthalpic contributions (Krasne & Eisenman, 1973). The recent crystallographic data of this enzyme have shown that sodium ion is coordinated with the oxygen of three carbonyl groups and two water molecules. This coordination is consistent with the observed loose selectivity of the cation-binding site. In fact, one of the general principles of Eisenman theory (Eisenman & Dani, 1987) predicts that cation-binding sites containing water are not very selective. Furthermore, a few weak dipole interactions between the cation and the protein, as those originated from carbonyl groups, might allow ions of different size to bind without large conformational changes. In contrast, the coordinated sphere of K^+ in dialkylglycine decarboxylase (Toney et al., 1993) and tryptophanase (Isupov et al., 1994; M. N. Isupov, personal communication) and that of Cs^+ in tyrosine phenol-lyase (Antson et al., 1994; A. A. Antson, personal communication) are formed by more strongly interacting residues (one carboxylate group, one hydroxyl moiety of a serine, three carbonyl oxygens, and only one water molecule in dialkylglycine decarboxylase; one carboxylate group, three carbonyl oxygens, and three water molecules in both tryptophanase and tyrosine phenol-lyase). This structural arrangement and the conformational changes observed upon cation substitution (Toney et al., 1993; Hoehenster et al., 1994) well explain the stronger ion selectivity observed in these enzymes.

Effect of Ions on the Dynamic Properties of the Enzyme. The phosphorescence decay of aromatic amino acids provides unique information on the dynamic properties of the protein (Strambini, 1989). Lifetimes of the excited triplet states are strongly decreased in fluid media since radiationless transitions to the ground state are increased. Thus, there is a direct correlation between lifetime and microviscosity of the protein matrix (Strambini & Gonnelli, 1985) that has been exploited for the characterization of the protein flexibility near Trp residues in response to the interaction with substrate, effector molecules, and metal ions (Cioni & Strambini, 1989; Cioni et al., 1989; Strambini & Gonnelli, 1990; Strambini et al., 1992a,b). The phosphorescence decay of Trp-177β, the only tryptophan present in tryptophan synthase, represents an intrinsic probe of the protein, well placed to report on structural changes at the subunit interface and, possibly, at the β-active site (Strambini et al., 1992a,b). The emission decay is clearly biphasic both in the absence and in the presence of monovalent cations, suggesting the existence of at least two protein conformations with equilibration time longer than the phosphorescence lifetimes. In the absence of cations, the conformation characterized by the shorter decay time, i.e., by higher flexibility, is predominant. When monovalent cations are present, there is a significant increase in the population of the conformation characterized by the longer lifetime. As a consequence, the τ_{av}, which measures the overall influence of a ligand on the dynamic properties of a protein, increases when monovalent cations are present. This effect, which is independent of the type of the bound ion, implies that monovalent cations stabilize a more compact conformation of the β-subunit. It is noteworthy that both the short and long lifetimes are not affected by cation binding. This finding suggests that cation binding does not alter the conformational properties of the protein but does alter a preexisting conformational equilibrium.

Effects of Ions on Catalytic Parameters. The presence of cations markedly improves the catalytic efficiency of the β-active site,[2] as revealed by the 20—40-fold increase of k_{cat}/K_M (Table 1). Remarkably, the ion effect on the rate of tryptophan synthesis does not strongly depend on ion type. This finding parallels the similar values of activation constants for different cations and the common conformational effects triggered by ions. However, some significant difference between ions can be detected by examining k_{cat} and K_M separately. In fact, K_M for indole decreases by reducing the size of the cation. From what it is known on tryptophan synthase catalysis, K_M values for indole reflect more than a single step. The complete process might include

[2] It should be noted that most of the early kinetic and spectroscopic studies on tryptophan synthase were carried out either in 100 mM potassium phosphate or in 50 mM bicine-NaOH buffer. Under such conditions, the enzyme is fully saturated by sodium or potassium. This fact and the observation that at room temperature the effects of Na^+ and K^+ are not very different can help to explain why in the past the influence of cations on the enzyme escaped notice.

noncovalent binding of indole to the α-active site, the travel down the tunnel, the binding to the β-active site, and its nearly irreversible reaction with the α-aminoacrylate (Lane & Kirschner, 1983b, 1991; Kirschner et al., 1991; Dunn et al., 1990; Anderson et al., 1991). Moreover, there is evidence that indole can gain access directly to the β-active site without passing through the tunnel, therefore without binding to the α-subunit (Dunn et al., 1990). When the effect of sodium concentration was investigated on the α-reaction in the $\alpha_2\beta_2$ complex, it was found that the K_M value of indole-3-glycerol phosphate increased with increasing concentration of the ion (Kirschner et al., 1991). Since indole-3-glycerol phosphate presumably shares with indole a subsite in the active site of the α-subunit, the strong decrease of K_M as a function of increasing sodium concentration that we have observed suggests that the effect of cations on the K_M of indole should be confined within the β-subunit. The existence of a preequilibrium of different PLP−serine−enzyme derivatives and their rate of interconversion must also be taken in account. These observations lead us to conclude that (i) the changes in K_M for indole due to cations can not be ascribed to a single step and (ii) the observed differences between cations imply differential effects on the individual catalytic steps. Similar arguments can be made for changes in k_{cat}. In fact, k_{cat} increases as a function of ion size with the exception of Li^+. From previous studies carried out in the presence of cations, it is known that at least three steps in the reaction catalyzed by tryptophan synthase have rate constants comparable to k_{cat}, i.e., they are partially rate-limiting at high indole concentration: deprotonation of the external aldimine, protonation of the quinonoid species obtained upon condensation of indole with the α-aminoacrylate, and release of L-tryptophan (Lane & Kirschner, 1991; Kirschner et al., 1991; Dunn et al., 1990; Anderson et al., 1991). Again, the observed differences between cations indicate distinct effects on one or more of these catalytic steps. This conclusion is supported both by data collected at 10 °C (Peracchi et al., 1994) showing a somewhat different order of cation activation and by the observation that various cations differently affect the equilibrium of intermediates formed in the presence of L-serine (see below). A study of the effect of cations on the elementary steps of α- and β-reactions has been recently reported (Dunn et al., 1994; Woehl & Dunn, 1995).

Effects of Ions on the Distribution of Catalytic Intermediates. Several PLP-dependent enzymes catalyzing β-elimination reactions are selectively activated by monovalent cations (Table 3). Some of these enzymes, although functionally similar, appear to be structurally unrelated (Alexander et al., 1994), a result that suggests a direct role of monovalent cations in catalysis. Suelter (1970) tentatively proposed a specific interaction between the metal ion and the common catalytic intermediate α-aminoacrylate. This hypothesis was neither further verified nor disproved. In the presence of L-serine, we have found that Cs^+, Rb^+, and Li^+ lead to the selective accumulation of a species absorbing at about 470 nm. This species might be a tautomeric form of the α-aminoacrylate, as suggested by Drewe and Dunn (1985). A band at 470 nm, formed in the β-elimination catalyzed by *O*-acetylserine sulfhydrylase, has also been assigned to the α-aminoacrylate Schiff base (Cook et al., 1992). However, Na^+ stabilizes the external aldimine and, in the presence of indole analogs, a quinonoid species (Dunn et al., 1994; Woehl & Dunn, 1995).

Table 3: Effect of Monovalent Cations on the Activity of PLP-Dependent Enzymes

					TS	
enzyme	DGD[a]	SDH[b]	TP[c]	TPL[d]	β_2 dimer[e]	$\alpha_2\beta_2$[f]
Li^+	↓		↔	↔	↑↑	↑↑
Na^+	↓	↑	↑	↓	↔	↔↑[g]
K^+	↑↑↑	↑	↑↑	↑↑	↑	↑
Rb^+	↑↑		↑↑	↑		↑↑
Cs^+	↔		↑	↑		↑↑↑
NH_4^+	↑↑	↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑

[a] DGD = dialkylglycine decarboxylase (Aaslestad & Larson, 1964; Hohenester et al., 1994). [b] SDH = serine dehydratase (Davies & Metzler, 1972). [c] TP = tryptophanase (Suelter & Snell, 1977). [d] TPL = tyrosine phenol-lyase (Demidkina & Myagkikh, 1989; Chen & Phillips, 1993). [e] TS = tryptophan synthase (Hatanaka et al., 1962). [f] TS = tryptophan synthase, this paper. [g] Dependent on temperature, ↑ = activator; ↓ = inhibitor; ↔ = no or small effect.

From these results it seems that cations play at least two distinct roles, a common one on conformational equilibria of the protein that might be linked to an overall increase of the rate of L-tryptophan synthesis and a specific one that leads to the stabilization of defined catalytic intermediates or local conformations.

Monitoring the Effects of Ions on the Crystalline Enzyme. Interesting suggestions on the molecular basis of cation−protein functional interaction might come from an X-ray analysis of the L-serine−enzyme complexes in the presence and absence of different monovalent cations. To this goal we have established that monovalent cations bind to the crystalline enzyme and elicit the same effects on the distribution of intermediates observed in solution. In particular, Na^+ and K^+ accumulate external aldimine, whereas Cs^+, Rb^+, and Li^+ stabilize the tautomeric form of the species absorbing at 470 nm. The dissociation constant of Cs^+ is similar in solution and in the crystalline state, whereas the dissociation constant for Na^+ is higher in the crystal than observed in solution. Functional differences between the crystalline and the soluble tryptophan synthase have previously been detected (Ahmed et al., 1987; Mozzarelli et al., 1989). The different amount of external aldimine and α-aminoacrylate present at equilibrium in the two physical states, under otherwise similar experimental conditions, can be accounted for by a different pH dependence of the equilibrium distribution of intermediates in the presence of monovalent cation in the crystal with respect to solution (Mozzarelli et al., 1991; Peracchi et al., 1994; Peracchi et al., unpublished observations).

On the basis of the distance between the cation-binding site and the active site, 11 Å in dialkylglycine decarboxylase and tryptophan synthase (David Davies, personal communication) and similar values in tyrosine phenol-lyase and tryptophanase, it seems likely that cations play a structural more than a direct catalytic role, stabilizing alternative enzyme and enzyme−intermediate conformations. Since in tryptophan synthase these conformations are characterized by distinct intersubunit interactions, the very fine regulatory tuning of α- and β-catalytic activities can be achieved by a variety of means, i.e., α- and β-subunit ligands, pH, and cations.

ACKNOWLEDGMENT

We are grateful to Dr. G. B. Strambini for the luminescence measurements, Dr. M. F. Dunn, Dr. D. R. Davies, Dr. A. A. Antson, and Dr. M. N. Isupov for communicating to

us their results prior to publication, and Dr. E. W. Miles for the kind gift of the *E. coli* strain containing the plasmid encoding the *S. typhimurium* gene.

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BI942957I