THE SUBSTRATE BINDING SITE SPECIFICITY OF A MODIFIED TRYPTOPHAN-SYNTHETASE β_2 SUBUNIT FROM *ESCHERICHIA COLI*

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1. Introduction

The β_2-subunit of *Escherichia coli* tryptophan-synthetase can be converted, by limited proteolysis, into an inactive protein which has kept several features of the native enzyme [1]. The overall hydrodynamic properties of the intact molecule, its ability to bind a substrate (indole) and the cofactor (pyridoxal-P), and even the capacity to form an enzyme-bound intermediate of the catalytic reaction are retained by the modified protein. This enzyme-bound intermediate, the 'aqua complex', is formed in the presence of the other substrate, L-serine and pyridoxal-P [2]. However, in the case of the native β_2 protein, the apparent dissociation constant for L-serine at 20°C and pH 7.8 is about 10^{-3} M while it is as high as 2×10^{-2} M in the case of the nicked β_2 protein [1]. In view of this 20-fold difference in the dissociation constants, and because of the very weak binding of L-serine to the nicked protein, one could wonder whether a specific binding site for this amino acid is or is not preserved during the proteolysis of the β_2 subunit and, consequently, whether it can be used as a significant probe of the protein conformation.

For this reason, the specificities of the intact and nicked β_2 proteins towards the amino acid involved in the formation of the aqua complex have been studied and are reported here.

2. Materials and methods

The crystallized tryptophan-synthetase β_2 subunit and the nicked protein were prepared as in [3]. The enzymatic activity and protein concentration were assayed as in [4] and [5], respectively.

The binding of L-serine to the intact or nicked protein was determined by measuring the fluorescence of the aqua complex, taking into account the dilution of the protein caused by the addition of the amino acid stock solution. The fluorescence was measured in a DC 3000/1 double monochromator spectrofluorimeter (CGA, Italy) equipped with a Servotrace FE recorder (SEFRAM, France). All measurements were performed at 20°C and pH 7.8 in 0.1 M potassium phosphate buffer containing 5×10^{-5} M pyridoxal-P and 2×10^{-3} M EDTA.

The same solvent was used to prepare stock solutions of the various amino acids used.

3. Results

3.1. *Binding of D-serine*

D-serine (5×10^{-2} M) was added to a solution of the nicked protein, and the fluorescence excitation spectrum of the protein was recorded. Figure 1b shows that no significant increase in the fluorescence could be observed, thus showing that D-serine is not able to ellicit the aqua complex (the slight increase in fluorescence could be shown to be due to a contamination of the D-serine with the L-isomer). However, the fluorescence of the aqua complex formed in the presence of 5×10^{-2} M L-serine (fig.1d) was very significantly decreased when the same concentration of D-serine was added to the solution (fig.1c). This result strongly suggests that, in spite of its inability to give rise to the fluorescent complex, the D-isomer is able to chase L-serine from the binding site.

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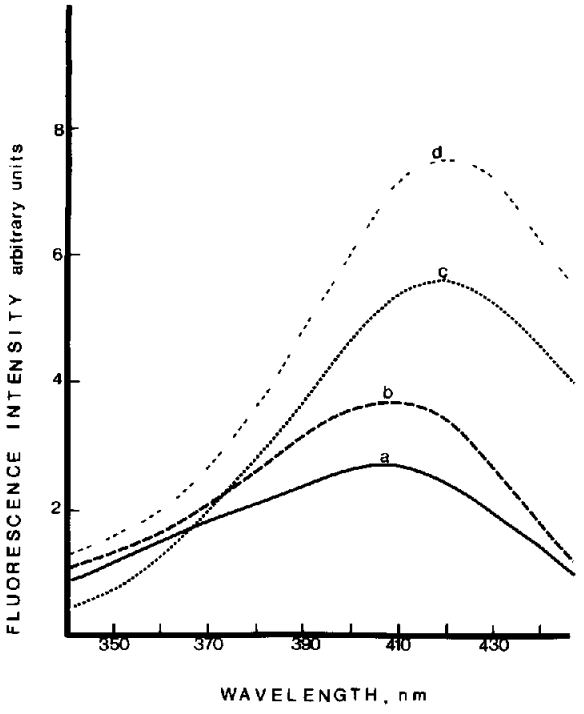

Fig.1. Effects of the serine isomers on the fluorescence spectrum of nicked β_2. The fluorescence excitation spectrum (λ emission = 510 nm) of the nicked β_2 protein (60 γ/ml) was recorder in 0.1 M potassium phosphate buffer, pH 7.8 containing 10^{-5} M pyridoxal 5'-phosphate and the following amino acids: (a) none; (b) D-serine 5×10^{-2} M; (c) D-serine 5×10^{-2} M and L-serine 5×10^{-2}; (d) L-serine 5×10^{-2} M.

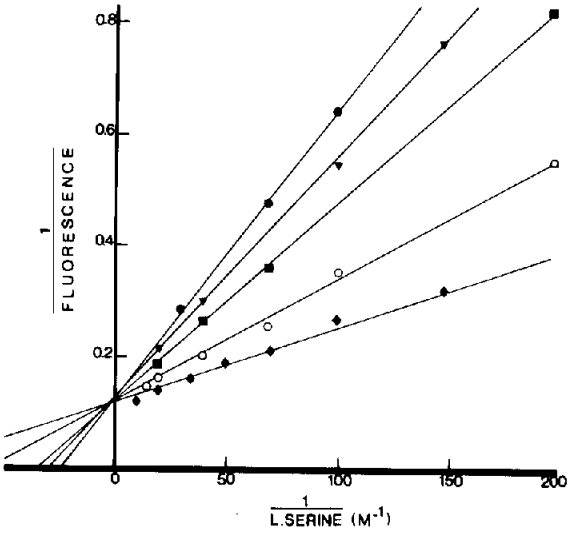

Fig.2. Effect of D-serine on the fluorescence of the aqua complex for nicked β_2. At each concentration of D-serine: 8×10^{-2} M (♦—♦); 10^{-2} M (○—○); 2×10^{-2} M (■—■); 3×10^{-2} M (▼—▼); 4×10^{-2} M (●—●); the increase in fluorescence intensity has been plotted as a function of the L-serine concentrations in double reciprocal coordinates. The excitation and emission wavelengths were, respectively, 440 nm and 510 nm and the protein concentration 90 γ/ml in 0.1 M potassium phosphate buffer pH 7.8 containing 10^{-5} M pyridoxal 5'-phosphate.

In order to determine the dissociation constant of the complex between D-serine and the nicked protein, the apparent dissociation constant for L-serine has been determined at various D-serine concentrations.

For this purpose, the fluorescence of the aqua complex formed as a function of the L-serine concentration was measured at various D-serine concentrations. The results were plotted in double reciprocal coordinates and are shown in fig.2. It can be seen that the straight lines obtained at the different D-serine concentrations intersect close to the axis of ordinates, suggesting that the bindings of the D- and L-isomers of serine are competitive. For a competitive binding, the apparent dissociation constant for L-serine at a given D-serine concentration, $K_{d_{app}}^{L\text{-ser}}$, should be a linear function of the D-serine concentration:

$$K_{d_{app}}^{L\text{-ser}} = K_d^{L\text{-ser}} \left[1 + \frac{(\text{D-serine})}{K_d^{D\text{-ser}}} \right]$$

where $K_d^{L\text{-ser}}$ and $K_d^{L\text{-ser}}$ are the dissociation constants for L- and D-serine respectively. This was verified as follows. The values of $K_{d_{app}}^{L\text{-ser}}$ at various D-serine concentrations were obtained by extrapolation of the straight lines in fig.2, and plotted as a function of the D-serine concentration. Figure 3 shows that the experimental points satisfactorily fall on a straight line, the slope of which yields the dissociation constant for D-serine. The value obtained, $K_d^{D\text{-ser}} = 1.5 \times 10^{-2}$ M, is not significantly different from that found for L-serine in the absence of D-serine: $K_d^{L\text{-ser}} = 1.3 \times 10^{-2}$ M [3]. It thus can be concluded that, though it does not

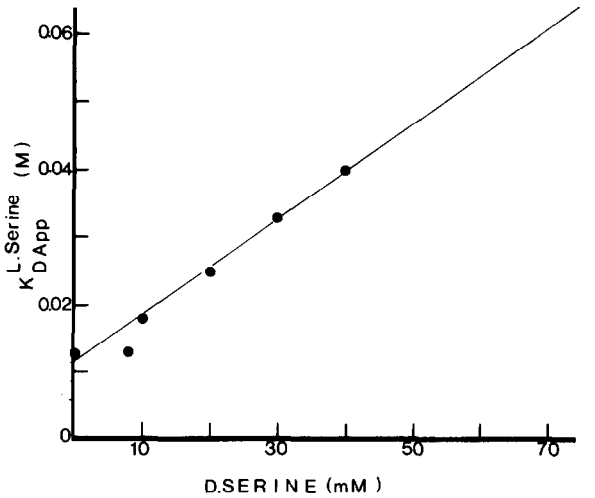

Fig.3. Determination of the dissociation constant for D-serine of nicked β_2. The apparent dissociation constants for L-serine obtained from fig.2 are plotted as a function of the D-serine concentration. The value obtained in the absence of D-serine is that in [1].

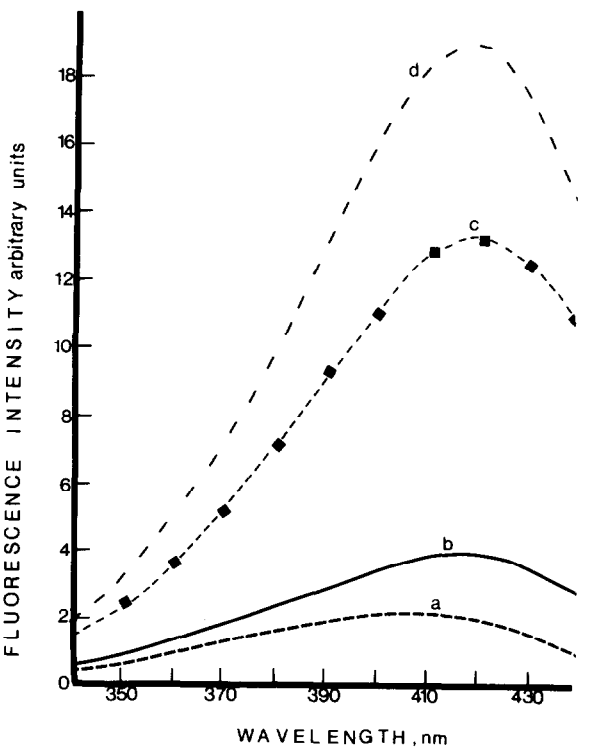

Fig.4. Effects of the serine isomers on the fluorescence spectrum of native β_2. The experimental conditions are as in fig.1 except for the β_2 protein and D-serine concentrations, which are, respectively, 35 γ/ml and 5×10^{-3} M.

give rise to the aqua complex, D-serine is bound to the nicked β_2 protein with the same affinity as L-serine in a competitive way.

Such a result was not expected since experiments, performed at lower amino-acid concentrations, had suggested that D-serine does not bind to the native β_2 protein [2]. It was therefore desirable to study the binding of D-serine to β_2 at high amino acid concentrations. First, it was confirmed that β_2 does not form the aqua complex even in the presence of 5×10^{-2} M D-serine. Second, a competition experiment was performed to see whether D-serine was able to displace L-serine from the aqua complex on native β_2. Only 5×10^{-3} M L-serine was used to saturate β_2 because of the higher affinity of intact β_2 for the L-isomer. Figure 4 shows that D-serine indeed decreases the fluorescence of the aqua complex elicited by L-serine. Assuming this decrease to be due to a strictly competitive binding of L- and D-serine, a dissociation constant for D-serine of 1.3×10^{-2} M could be computed.

It therefore can be concluded that D-serine binds with the same affinity to the intact and the nicked β_2 protein, without giving rise to the fluorescent aqua complex.

3.2. *Binding of L-alanine*

A similar study has been performed with L-alanine. This amino acid produced no change in the fluorescence of the pyridoxal-P bound to the nicked β_2 protein. Moreover, at a concentration of 5×10^{-2} M, it did not modify the fluorescence of the aqua complex formed by the nicked protein in the presence of 5×10^{-2} M L-serine. This indicates that L-alanine does not bind to the nicked protein.

The same conclusion had been reached earlier for the native protein [2].

3.3. *Binding of L-phenylalanine*

The interaction of L-phenylalanine to the nicked protein was next investigated. This aromatic amino acid was chosen because it was qualitatively known to bind to the intact β_2 subunit.

When L-phenylalanine (5×10^{-2} M) was added to

the nicked β_2 protein, the fluorescence of the aqua complex did not appear. However, a slight blue shift of the excitation spectrum of the enzyme-bound pyridoxal-P could be observed: the maximum was shifted from about 410 nm to about 400 nm. This already suggested that L-phenylalanine is indeed able to bind to the nicked protein.

This was further verified by a competition experiment analogous to that described above for D-serine. When, to the aqua complex formed in the presence of 5×10^{-2} M L-serine, L-phenylalanine (5×10^{-2} M) was added, the fluorescence of the complex was diminished by 60%. Assuming a strictly competitive binding of these two amino acids, a dissociation constant of 6×10^{-3} M could be computed for L-phenylalanine.

Similar experiments were also performed to quantify the binding of L-phenylalanine to the intact β_2 protein, except the concentration of L-serine in the competition experiment was reduced to 5×10^{-3} M. The same results as above were obtained: L-phenylalanine did not give rise to the fluorescence of the aqua complex, produced a blue shift of the fluorescence of the enzyme-bound cofactor, and competed with L-serine with a dissociation constant of 9×10^{-3} M, not significantly different from that found above in the case of the nicked protein.

4. Discussion

The results above can be very simply summarized as follows: for each amino acid analogue which was studied, the binding constant for the intact and the nicked protein was found to be, within experimental error, the same. Similarly, each amino acid produced the same effect on the fluorescence of these two protein species and only L-serine was indeed found to ellicit the 'aqua complex'. It thus can be concluded that the specificity of the L-serine binding site has been preserved upon converting β_2 into its nicked derivative, and that the integrity of this binding site in the nicked protein indeed confirms that the nicked protein has kept a conformation closely resembling that of the native enzyme.

In addition, some insight into the binding of the amino acid to the protein has been obtained through the results reported above. That bound D-serine does not modify the fluorescence excitation spectrum of the protein–pyridoxal-P complex indicates that this amino acid cannot form a Schiff base with the enzyme bound cofactor, while it is well known that the L-isomer does form a Schiff base. Thus, D-serine appears to sit at the binding site with its α-amino group located at a different position than that of the amino group of bound L-serine. Yet, the L- and D-isomers bind equally well to the nicked protein. It therefore can be concluded that the α-amino group of the substrate does not play an important role in the binding of the amino acids.

Surprisingly, the formation of the Schiff base between L-serine and the cofactor bound to the protein also does not appear to significantly contribute to the overall free energy of binding of L-serine to the nicked protein since D-serine, which does not form the Schiff base, binds as well as L-serine.

It thus appears that the specificity of the binding to β_2 primarily concerns the substitution on the β-carbon of the amino acid substrate as demonstrated by the widely different binding properties of L-alanine, L-phenylalanine and D-serine described above. Only, in the case of L-serine, the single real substrate of β_2 examined in this study, has a difference been observed between the nicked and the intact β_2-protein, and this difference deals with both the activity and binding constant of the two proteins. It is conceivable that the inactivation and the decrease of the affinity for L-serine which occur upon nicking the β_2-protein involve the same structural modification of the active site. The characterization of this modification would certainly be of high interest in understanding the configuration and mode of catalysis of the active site.

Acknowledgements

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