Volume 320, number 3, 224–228 FEBS 12302 April 1993

Nicking of the tryptophan synthase β_2-subunit at Glu-296 prevents the conformational change undergone on binding the α-subunit

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Received 19 February 1993

Using a monoclonal antibody as conformational probe it has been shown that the weakly active nicked-β2 dimer of tryptophan synthase generated by proteolytic cleavage at Glu-296, does not undergo on association with α subunit a conformational change known to occur in intact β2 subunit. This α induced conformational change is also prevented in intact β2 by the coenzyme pyridoxal-5′-phosphate when the substrate L-serine is absent

Conformational change; Tryptophan synthase; Monoclonal antibody; Proteolytic cleavage; Ligand binding

1. INTRODUCTION

In a recent study Kaufmann et al. [1] have shown that nicking the tryptophan synthase holo β2 subunit from *E. coli* at the C-terminal side of Glu-296 leads to an active species still capable of interacting with α subunits, while it is known that the limited proteolysis of β2 with trypsin leads to a nicked protein which is inactive and incapable of binding α subunits [2,3]. As shown in the X-ray structure performed with the largely homologous enzyme from *Salmonella typhimurium* [4] the cleavage sites are located in a region (hinge region) corresponding to a loop without defined secondary structure. Several residues of the interface between the α and β subunits are carried by the 11 amino acid peptide that is lost during the proteolytic cleavage of β2 by trypsin. This may account for the lack of association between α and trypsin nicked β2. The trypsin and endoproteinase Glu-C nicked β2 retain a quasi native structure, remain dimeric and are able to bind the coenzyme pyridoxal-5′-phosphate and the substrate L-serine. However, while binding of pyridoxal-P to intact β2 is cooperative [5] and seems to obey the two-state model of Monod et al. [6], the fixation of the coenzyme to nicked β2 is no more cooperative [1,7]. Tschopp and Kirschner [7] had concluded that the nicked protein is 'frozen' in a state with low affinity for pyridoxal-P. The results obtained by Chaffotte and Goldberg [8] on the accessibility to water of the tryptophan residues in trypsin nicked β2 and those obtained by Djavadi-Ohaniance et al. [9] by probing trypsin nicked β2 with monoclonal antibodies supported this hypothesis. It is also known, from spectroscopic [5,7,10–12] and immunochemical [9] investigations, that the β2 subunit undergoes conformational rearrangements upon binding of the coenzyme pyridoxal-5′-phosphate, the substrate L-serine, or the α subunit.

In order to gain a better understanding of the mechanism of inactivation of β2 by limited proteolysis, and more specifically of the effect of the proteolysis on the conformational changes of β2, it was interesting to investigate if the α induced conformational change observed in native β2 also occurs in Glu-C nicked β2. To do this, we chose as conformational probe a monoclonal antibody directed against native β2 that was previously obtained and characterized in our laboratory [13,14]. This monoclonal antibody (93-6) was a good candidate because of the following reason.

Though β2 is a symmetrical dimer [4] and therefore should carry two identical binding sites per β2, only one Fab 93-6 can bind per β2 [9] probably because of steric hindrance as suggested by small-angle X-ray scattering studies [15]. However it was observed that in the $\alpha 2\beta 2$ complex, the second binding site for this antibody became accessible [16]. This unmasking of the second epitope on β2 was interpreted as reflecting a major conformational rearrangement that occurs upon binding of α to β2.

The present paper reports investigations, carried out with monoclonal antibody 93-6 on the conformational change undergone by apo and holo β2 upon association

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Abbreviations α, β2, α or β2 subunit of *Escherichia coli* tryptophan synthase; reduced β2, $NaBH_4$ reduced holo β2; Ig, immunoglobulin, mAb, monoclonal antibody; SDS, sodium dodecyl sulfate, PEG 6000, polyethyleneglycol with M_r = 6,000; Pyridoxal-P, pyridoxal 5′-phosphate; Tryptophan synthase, EC 4.2.1.20.

with α, either in the absence or in the presence of the substrate L-serine, and on the effects of nicking β2 with endoproteinase Glu-C on this conformational change.

2. MATERIALS AND METHODS

2.1. *Buffers and reagents*

The standard buffer was 0.1 M potassium phosphate, pH 7.8, and 2 mM (ethylenedinitrilo)tetraacetic acid. If present, the concentration of pyridoxal-P* (from Sigma) was 0.1 mM.

2.2. *Proteins*

Preparation of the α subunit was performed as described previously [17]. Apo β2 was purified as described by Högberg-Raibaud and Goldberg [3]. Holo β2 was obtained by incubation of apo β2 in the presence of 0.1 mM pyridoxal-P

Nicked β2 was prepared using Endoproteinase Glu-C from *Staphylococcus aureus* V-8 strain, purchased from Boehringer (Mannheim). The preparation and isolation of the single cut nicked β2 was performed as described by Kaufmann et al. [1]

The protein concentrations were determined by measuring the absorbance at 280 nm. The extinction coefficients were 0.6, 0.65, 0.46 and 1.4 for apo β2, holo β2, α and IgG, respectively [18,19]. Monoclonal antibody mAb 93-6 was prepared as previously described [13,14].

2.3. *Measurement of the affinity constants*

The affinity constant (K_A) in solution was determined by the method previously described [20]. Briefly, a constant amount of the antibody was incubated with a varying amount of the antigen, until equilibrium was reached. The quantity of free antibody was monitored by a classical indirect ELISA.

2.4. *Turbidity measurements*

To monitor the precipitation of the mAb 93-6 immune complexes, the turbidity of the mixture was measured in 1 cm cuvettes at 500 nm At this wavelength no absorbance in the absence of precipitation was observed.

2.5. *Precipitation of the immune complex with an anti-mouse antibody*

The immune complex was formed by incubation of α and β2 (100 μM and 5 μM, resp.) for 15 min at room temperature (RT), subsequent addition of the mAb (93-6, 10 μM) and incubation for a minimum of 6 h at RT. Then rabbit immunoglobulins anti-mouse Ig (Biosys-France) were added (10 μM). After 6 h incubation at RT, precipitation was induced by addition of an equal volume of 7.5% PEG 6000 (Riedel-De Haën, Germany) and incubation for 1 h at 4°C. After centrifugation for 10 min at 13,000 × *g* and 4°C, the pellet was rinsed twice with water to remove the remaining PEG 6000 and suspended in sample buffer without β-mercaptoethanol for further SDS gel electrophoresis.

2.6. *SDS gel electrophoresis*

The electrophoresis was performed using the Phast System of Pharmacia (Uppsala, Sweden). The gels used were 8–25% acrylamide gradient Phast gels, the sample buffer did not contain β-mercaptoethanol

3. RESULTS

As reported before, only one Fab of monoclonal antibody 93-6 can bind per β2 dimer [9] and the binding of α to apo β2 is accompanied by the unmasking of the second antibody binding site on the antigen [16]. Consequently, the binding of two bivalent Ig 93-6 molecules to apo α2β2 leads to the formation of a high molecular weight complex and to the precipitation of the antigen–antibody complex. On the other hand when only one site is accessible, no precipitation occurs. We therefore chose to use the precipitation phenomenon as a measure of the number of binding sites available on β2. The precipitate was evaluated spectroscopically by measuring the turbidity of the mixture at 500 nm.

3.1. *The α-induced conformational change analyzed in native β2*

First, apo β2 (5 μM) was incubated for 15 min with α (20 μM) to allow the formation of the α2β2 complex. These concentrations of α and β2 resulted in complete saturation of β2 since the equilibrium dissociation constant of α2β2 is about $2 \cdot 10^{-7}$ M [21]. Then Ig 93-6 (10 μM) was added and the turbidity of the solution was measured at different time intervals. As shown in Fig. 1a, a plateau was reached after 1 h. In order to check the nature of the polypeptides present in the precipitate, the mixture containing α, β2 and Ig 93-6, incubated together for 1 h, was centrifuged for 10 min at 5,000 × *g*. The pellet was dissolved in the sample buffer and analyzed by SDS gel electrophoresis. The pattern obtained after staining with Coomassie blue showed the bands corresponding to the IgG of the antibody and to the α and β subunits. When Ig 93-6 and apo β2 at the same concentrations as above were mixed in the absence of α, no precipitation was detected even after 3 h of incubation (data not shown). These observations confirm that, in the presence of α only, a precipitate containing α, β and γ 93-6 was formed and that the formation of the precipitate was completed within about 60 min. To investigate the effect of the specific ligands pyridoxal-P and L-serine on the changes undergone by β2 upon

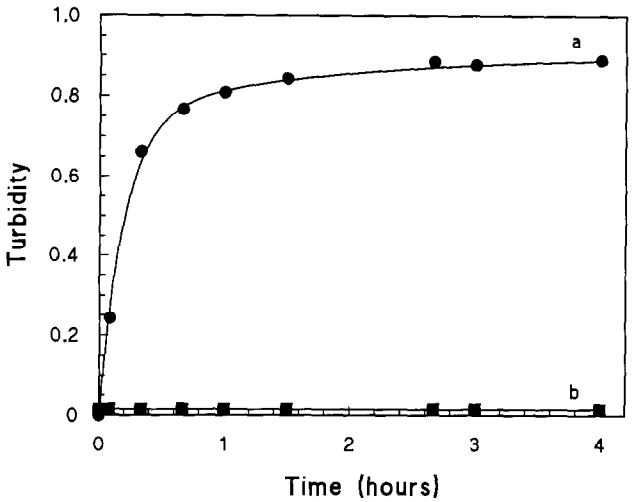

Fig. 1 Precipitation of α2β2 monitored after addition of the mAb 93-6 in the absence or presence of pyridoxal-P, and of L-serine. β2 (5 μM) was incubated with α (20 μM) and Ig 93-6 (10 μM) was added after 15 min. The turbidity of the mixture was measured at 500 nm immediately and at different time intervals (a) apo α2β2 or holo α2β2 in the presence of 0.1 M L-serine. (b) holo α2β2 without serine.

binding of α, we studied the influence of these ligands on the extent and kinetics of the precipitation.

(a) Effect of the coenzyme, pyridoxal-P To analyze the effect of pyridoxal-P on the changes induced by α on β2, the precipitation was measured after the addition of Ig 93-6 (10 μM) to a mixture of holo β2 (5 μM) and α (20 μM). In contrast to the observation made with apo α2β2, no precipitation was observed with the holo form of the protein (Fig. 1b). Thus the binding of the coenzyme was shown to prevent the α-dependent conformational rearrangement of the β2 subunit responsible for the unmasking of the second binding site for monoclonal antibody 93-6.

(b) Effect of the substrate L-serine It is known that apo β2 does not bind L-serine and that the binding of L-serine to holo β2 is accompanied by a conformational change in β2 [9,11]. To check whether or not L-serine might also affect the conformation of the holo α2β2 complex, we probed its effect on the reactivity of holo α2β2 with the antibody 93-6. Adding Ig 93-6 (10 μM) to a mixture of holo β2 (5 μM) and α (20 μM) containing 0.1 M L-serine resulted in a precipitation equivalent to that observed in the case of apo α2β2 (Fig. 1a). Thus, the presence of L-serine released the constraint exerted on β2 by the coenzyme that prevented the precipitation in the absence of serine.

In holo β2 as well as in holo α2β2, a Schiff's base is formed between the ε-amino group of lysine 87 and pyridoxal-P. In the presence of L-serine, this Schiff's base is broken, and a new one is formed between the α-amino group of the substrate and the coenzyme. The fact that the second binding site for the monoclonal antibody 93-6 is unmasked by the fixation of α on apo β2 and holo β2 with L-serine, but not on holo β2 in the absence of L-serine, suggested that the presence of the Schiff's base between lysine 87 and pyridoxal 5'-phosphate might prevent the conformational change induced by α. If this were the case, no precipitation by monoclonal antibody 93-6 of sodium borohydride reduced holo α2β2 should occur in the presence of L-serine, since the reduction of the Schiff's base renders irreversible the covalent bond between pyridoxal-P and lysine 87. This was checked by adding Ig 93-6 (10 μM) to a mixture of reduced holo β2 (5 μM) and α (20 μM) containing 0.1 M L-serine. No precipitation was observed, in contrast to the case of non reduced holo α2β2, with L-serine (Table I). This suggested that the Schiff's base may play an important role in preventing the α-induced conformational change in the case of holo β2.

3.2. *The α-induced conformational change analyzed in Glu-C nicked β2*

It was interesting to see if the Glu-C nicked β2 protein, which retains some residual activity and the capacity to bind α subunit [1], also retains the ability to undergo a conformational change upon binding α. To investigate this through the immunochemical approach described above, it was first necessary to determine the affinity constant of monoclonal antibody 93-6 for Glu-C nicked β2 to ensure that the proteolytic cleavage did not result in a drastic modification of the epitope.

The affinity constant in solution was therefore measured using the ELISA method previously described [20]. The affinity constants of monoclonal antibody 93-6 were found to be 2.2, 3 and $4 \cdot 10^8$ M^{-1}, respectively, for the apo, holo and holo with serine forms of Glu-C nicked β2. These values were not significantly different from one another, and were close to those reported [9] for trypsin nicked apo or holo β2 and for intact holo β2 with or without L-serine ($K_A = 4.2 \cdot 10^8$ and $5.9 \cdot 10^8$ M^{-1}, respectively).

The fact that the proteolytic cleavage did not affect significantly the binding of monoclonal antibody 93-6 to nicked β2, allowed us to investigate the eventual change induced by α on Glu-C nicked β2 with the same precipitation test as that used for intact β2. However, since the association constant of α to Glu-C nicked β2 is only about $2 \cdot 10^{-5}$ M^{-1} [1], we used a 100 μM concentration of α, high enough to saturate nicked β2 (5 μM).

Neither for nicked apo β2 nor for nicked holo β2 or nicked holo β2 with serine could precipitation be detected after the addition of α (100 μM) and Ig 93-6 (10 μM) (Table I) suggesting that the second epitope did not become accessible upon binding of α. To ascertain this conclusion, it was however important to verify that the ternary complex between α, nicked β2 and one molecule of Ig 93-6 was indeed formed. To do this, an immunoprecipitation experiment was carried out as described in Section 2 according to the following rationale: nicked β2, α subunit and Ig 93-6 were mixed together, and the immunoglobulin (free or bound to nicked β2 or nicked α2β2) present in the solution was specifically precipitated with a rabbit anti-mouse antibody. The nature of the polypeptide chains present in the immune complex was then analyzed by gel electrophoresis. As control, similar experiments were performed without any β2 or with native β2 instead of nicked β2.

The gel shown in Fig. 2 revealed that α was indeed

Table I

Precipitation of β2 with monoclonal antibody 93-6 observed in the absence or presence of the specific ligands or after nicking the protein with endoproteinase Glu-C

Ligands	mAb 93-6 with			
	β_2	$\alpha_2\beta_2$	Nicked-β_2	α_2 Nicked-β_2
None	–	+	–	–
Pyridoxal-P	–	–	–	–
Pyridoxal-P and serine	–	+	–	–

+, presence of precipitation monitored as described in Fig. 1, –, no precipitation.

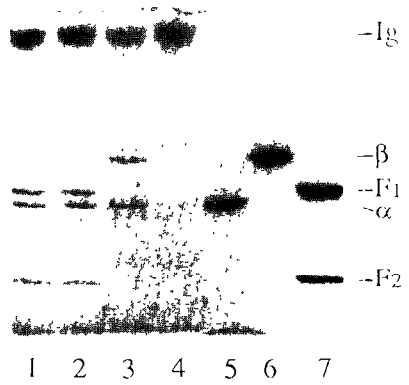

Fig. 2. SDS gel electrophoresis of the immune complexes after precipitation with an anti-mouse antibody. As described in Section 2, $\beta 2$, α, and the monoclonal antibody were mixed and the immune complex was precipitated with an anti-mouse Ig antibody. The pellet obtained after centrifugation was analyzed by gel electrophoresis in the presence of SDS. Slot 1: nicked holo $\alpha 2\beta 2$. Slot 2: nicked holo $\alpha 2\beta 2$ in the presence of 0.1 M L-serine. Slot 3: holo $\alpha 2\beta 2$. Slot 4: without $\beta 2$. Slots 5, 6, 7: α, $\beta 2$, nicked $\beta 2$ as markers.

present in the immune complex in the experiments with nicked holo $\beta 2$ without or with serine (slots 1 and 2) as is the case for intact holo $\beta 2$ (slot 3). The same result (not shown) was obtained with nicked apo $\beta 2$. On the other hand no band corresponding to α could be detected in the experiment where $\beta 2$ was absent (slot 4) indicating that contamination of the pellet through nonspecific binding of α was negligible. These results clearly showed that a ternary complex containing α, nicked $\beta 2$ and Ig 93-6 could be formed, but did not precipitate. This confirmed that the second binding site for monoclonal antibody 93-6 was not unmasked upon association of α to Glu-C nicked apo $\beta 2$ or holo $\beta 2$ plus serine, unlike what happens for native apo $\beta 2$ and holo $\beta 2$ plus serine.

4. CONCLUSIONS

It has been previously shown that the *E. coli* tryptophan synthase $\beta 2$ dimer which should carry two epitopes for each monoclonal antibody, binds only one Fab of mAb 93-6 probably because of steric hindrance [15], while $\alpha 2\beta 2$ binds two Fab 93-6 [16]. In the quaternary structure of the tetrameric complex of $\alpha 2\beta 2$ [4] the two β subunits which are at the center of the complex interact over a broad nearly flat surface through which a dyad axis of symmetry passes. The unmasking of the second Fab 93-6 binding site on apo $\beta 2$ upon binding α could originate from a change in the relative position of the two β chains that carry the epitopes of the mAb promoting a significant rearrangement in the quaternary structure of $\beta 2$.

The results reported in this paper first show that the binding of the coenzyme pyridoxal-P and of the substrate L-serine has an important influence on the α-induced conformational rearrangement of the $\beta 2$ subunit. Holo $\beta 2$ in contrast to apo $\beta 2$, is unable to undergo the change leading to the unmasking of the second binding site for the antibody 93-6. However, holo $\beta 2$ in the presence of L-serine behaves as apo $\beta 2$ and can bind two Fab 93-6 when associated to α. Thus L-serine releases the constraint imposed to $\beta 2$ by the coenzyme. The finding that L-serine does not release this constraint in reduced-holo $\beta 2$ suggests that the Schiff's base between the coenzyme and the protein plays a role in controlling the balance between the two conformational states of $\beta 2$. During the catalytic cycle of tryptophan synthase, the enzyme constantly turns over from a state with the Schiff's base with Lys-87 and no bound serine, to a state with bound serine and no Schiff's base between the coenzyme and Lys-87. It therefore seems very likely that the $\beta 2$ subunit keeps oscillating between the two conformational states identified with mAb 93-6.

As reported by Kaufmann et al. [1], the proteolytic cleavage of holo $\beta 2$ subunit at Glu-296 leads to a partly active species capable of weakly interacting with α subunits (Kaufmann et al., 1991). $\beta 2$ cleaved at other positions in the hinge region (Lys-272, Arg-275, Lys-283) is enzymatically inactive and cannot associate with the α subunit [2,3].

We have shown here that neither for Glu-C nicked apo $\beta 2$ nor for nicked holo $\beta 2$ or nicked holo $\beta 2$ with L-serine, does the second binding site of mAb 93-6 become readily accessible after the association of α, indicating that the single cut at Glu-296 prevents the protein from undergoing the conformational rearrangement observed with the intact $\beta 2$ upon the association of α. That nicked $\beta 2$ which has a reduced enzymatic activity, fails to readily undergo the α-induced conformational rearrangement identified with monoclonal antibody 93-6, suggests that this rearrangement may be an essential part of the catalytic mechanism of the $\alpha 2\beta 2$ complex.

Acknowledgements: The authors thank Pr. Peter Bartholmes for helpful discussions and his interest in this work. This work was supported by funds from the Institut Pasteur, the Centre National de la Recherche Scientifique (CNRS URA 1129) and the Université Paris 7. One of the authors (H.J.L.) was supported by a grant from the Deutsche Forschungsgemeinschaft (Ba 527/6-1) and a Deutscher Akademischer Austauschdienst short-term Fellowship.

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