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Limited Proteolysis of the β_2 -Dimer of Tryptophan Synthase Yields an Enzymatically Active Derivative That Binds α -Subunits[†]

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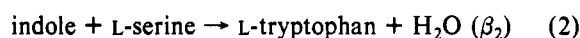
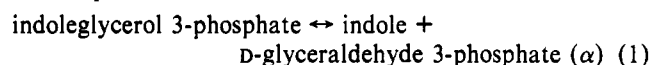
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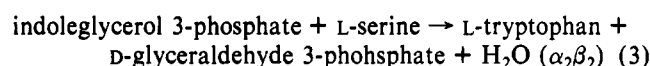
ABSTRACT: Proteolytic modification of tryptophan synthase holo- β_2 -subunit from *Escherichia coli* at the C-terminal side of E-296 leads to an active species (E-296-nicked holo- β_2) capable of interacting with α -subunits. Although this heterologous subunit interaction is rather weak, it induces an increase in catalytic efficiency in E-296-nicked holo- β_2 by a factor of about 150. Correspondingly, enzymatic activity of α -subunits is enhanced 180-fold. This is in striking contrast to the findings from earlier reports which demonstrated that proteolytic derivatives modified at other positions in the "hinge region" embedded in the C-domain of the β_2 -subunit (K-272, R-275, and K-283) are enzymatically inactive and cannot associate with α -subunits. The equilibrium binding curve for the cofactor pyridoxal 5'-phosphate to E-296-nicked apo- β_2 is hyperbolic (i.e., noncooperative), yielding an apparent microscopic dissociation constant, K_d , of 5×10^{-6} M. This value closely resembles the low-affinity dissociation constant of cooperative cofactor binding to the native β_2 -subunit, indicating that the conformational interactions between structural domains in the modified β -protein seem to be disturbed considerably.

Modification of proteins by limited proteolysis is a powerful tool to evaluate structure-function relationships as well as elementary steps in the pathway of protein folding. Regions of subunit interaction in complex protein structures may be identified, and it is possible to map the sections of the protein sequence responsible for biological activity. This technique was applied to tryptophan synthase from *Escherichia coli* (EC 4.2.1.20) which is a holoenzyme complex composed of a tightly associated central β_2 -dimer combined with two peripheral α -subunits (Yanofsky & Crawford, 1972; Miles, 1979; Miles et al., 1987).

Two partial reactions:



are combined to the overall reaction



The respective catalytic efficiencies of the isolated α - and β_2 -subunits for reactions 1 and 2 are only $\leq 2\%$ as compared to the enzymatic activities in the completely associated $\alpha_2\beta_2$ complex catalyzing the overall reaction 3. This indicates the existence of binding sites with strong allosteric control. As known from recent X-ray crystallographic studies performed with the largely homologous enzyme from *Salmonella typhimurium* (Hyde et al., 1988), the tertiary structure of the

β_2 -protomer includes a 54-residue stretch ("hinge region") without defined secondary structure which is located near the interface between the α - and β_2 -subunits (amino acids 257-310). Although this "hinge region" was described previously as the connecting peptide between two independent folding regions F1 and F2 of the β_2 -subunit (Högberg-Raubaud & Goldberg, 1977a,b), X-ray structure clearly demonstrated that this peptide is an internal part of the C-domain (Hyde et al., 1988). It contains three well-known sites for proteolytic cleavage (K-272, R-275, and K-283, Figures 1 and 2). Limited proteolysis using trypsin or endoproteinase Lys-C causes the loss of 11 amino acids H-273-K-283, here named "Goldberg-loop" (Högberg-Raubaud & Goldberg, 1977a,b), whereas endoproteinase Arg-C produces a single cut at R-275 (Ahmed et al., 1986). In both cases, the resulting protein is inactive and incapable to bind α -subunits, indicating the important role of that particular region in the β_2 -subunit. Moreover, it is evident from the above-mentioned X-ray structure that the Goldberg-loop is associated with a region of multiple turns in the α -subunit (Figure 1).

Since limited proteolysis of the α -subunit by trypsin yields an active derivative which binds to the β_2 -dimer (Miles & Higgins, 1978; Higgins et al., 1979), we were led to assume that modification by other proteases cleaving the β_2 -subunit at different sites in the hinge region could result in derivatives with similar residual function. In this work, we investigate a derivative proteolyzed at position E-296 which is distant from the Goldberg-loop, and does not directly interact with the opposite α -subunit. Proteolysis at E-296, subsequent purification of F2 fragments, and an antibody binding study of three monoclonal antibodies directed to the C-terminal domain were published recently (Friguet et al., 1989).

EXPERIMENTAL PROCEDURES

Chemicals. Pyridoxal 5'-phosphate and bovine serum albumin were obtained from Serva (Heidelberg), α_2 -macro-

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FIGURE 1: Stereo image of the α - β contact region of tryptophan synthase from *S. typhimurium* which is largely homologous to the *E. coli* enzyme. Four sites of proteolytic cleavage are marked with the corresponding amino acid numbers. α -Chain (light dashes); β -chain (light solid line); hinge region (dark dashes); and Goldberg-loop (dark solid line).

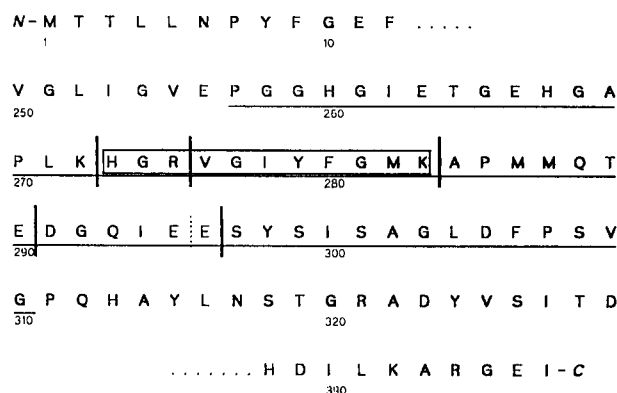


FIGURE 2: Partial primary sequence of the β -chain as derived from nucleotide sequence according to Crawford et al. (1980). Location of proteolytic sites, vertical bars and vertical dots; hinge region, P-257-G310 (underlined); Goldberg-loop, H-273-K283 (box).

globulin was obtained from Boehringer (Mannheim), urea was purchased from Riedel de Haen (Seelze), and all other chemicals were of A-grade purity from Merck (Darmstadt). Quartz bidistilled water was used throughout.

Indoleglycerol 3-phosphate was synthesized from fructose 1,6-bisphosphate and indole in a sterilizable hollow-fiber membrane reactor from AKZO (Oberburg) with an intracapillary volume of 16 mL, using aldolase, triosephosphate isomerase, and tryptophan synthase.

Enzymes. The isolated α - and holo- β_2 -subunits (with two molecules of the cofactor pyridoxal 5'-phosphate bound per dimer) of tryptophan synthase from *Escherichia coli* were purified as described previously (Kirschner et al., 1975; Bartholmes et al., 1976). Endoproteinase Glu-C from *Staphylococcus aureus* V-8 strain, rabbit muscle aldolase, and rabbit muscle triosephosphate isomerase were obtained from Boehringer (Mannheim).

Buffers. Buffer A was 0.1 M potassium phosphate, pH 7.8, 2 mM (ethylenedinitrilo)tetraacetic acid (EDTA),¹ and 8 ×

10⁻⁵ M pyridoxal 5'-phosphate. Buffer B consisted of 0.1 M potassium phosphate, pH 7.8, and 2 mM EDTA.

Limited Proteolysis and Purification of E-296-Nicked β_2 . Endoproteinase Glu-C was used to obtain specific cleavage at the carboxy-terminal side of glutamyl residues in the hinge region of β_2 (Houmard & Drapeau, 1972). Proteolysis was performed at 37 °C with 1 mg of holo- β_2 /mL of buffer A. Endoproteinase Glu-C concentrations varied between 0.06 and 6 μ g/mL, and incubation times were 10 min (6 μ g/mL) or 16 h (0.06 μ g/mL) for complete transformation of native β_2 into the proteolyzed species. For SDS-PAGE analysis, aliquots of the incubation mixture were added to sample buffer after increasing time intervals, and the reaction was stopped by transfer to liquid nitrogen. In all other cases, inhibition of proteolysis was achieved by adding 5 units of α_2 -macroglobulin/ μ g of protease, followed by 2-h incubation at 37 °C in order to allow all protease molecules to be trapped by this effective inhibitor (Barrett, 1981). Subsequently, 8 mL of E-296-nicked holo- β_2 was purified by anion-exchange FPLC (Pharmacia, Uppsala) using 90 mL of a linear sodium chloride gradient (0–300 mM in buffer B) on a Mono-Q HR 5/5 column (volume = 1 mL) with a flow rate of 1 mL/min. Fractions of 3 mL were collected, and E-296-nicked holo- β_2 , as determined by SDS-PAGE, was pooled, dialyzed against buffer A, and concentrated in Centricon 10 microconcentrators (Amicon, Witten) up to final concentrations of about 5 mg/mL. E-296-nicked apo- β_2 was prepared by adding a 10-fold excess of hydroxylammonium chloride followed by extensive dialysis against buffer B (De Moss, 1962).

Purification of F1a, F1b, and F2 Fragments. FPLC fractions of peak 2 (Figure 4) (for isolation of F1b and F2) and FPLC fractions of peak 4 (for isolation of F1a and F2) were separately pooled and concentrated to a volume of 1 mL by using Centricon 10 microconcentrators (Amicon, Witten). Solid urea was added in order to obtain a final concentration of 6 M, and the N-terminal fragments (F1a and F1b) were separated from the C-terminal F2 peptide by gel filtration over Sephacryl S-200 eluted with buffer B plus 6 M urea. Fractions containing the individual chains as judged by SDS-PAGE were pooled, and the fragments were transferred into 10 mM triethanolamine hydrochloride/10 mM β -mercaptoethanol, pH 7.8, by gel filtration using Sephadex G-25M PD-10 columns (Pharmacia, Uppsala). Finally, the preparations were freeze-dried by using UNIVAPO 150 H/UNICRYO MC 2C equipment (Fröbel, Lindau) and stored at -20 °C prior to use for sequence analysis.

¹ Abbreviations: ³¹P NMR, phosphorus-31 nuclear magnetic resonance; EDTA, (ethylenedinitrilo)tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; E-296-nicked β_2 , β_2 -dimer of tryptophan synthase from *E. coli*, proteolytically modified at the C-terminus of E-296 [notation with respect to the nucleotide sequence as taken from Crawford et al. (1980)]; it consists of four tightly associated peptides (T-2-E-296/S-297-I-397)₂ named (F1a/F2)₂ containing the same number of residues as the native β -dimer (T-2-I-397)₂.

Protein Concentrations. The amount of protein was measured as described earlier using bovine serum albumin as a protein standard (Bradford, 1976).

Enzyme Measurements. Enzymatic activities of holo- β_2 and E-296-nicked holo- β_2 were determined in the absence and in the presence of a 3-fold excess of α -subunits by monitoring reaction 2 at 289 nm (Faeder & Hammes, 1970). Enzymatic activity of α -subunits (reaction 1) was measured by using a glyceraldehyde-3-phosphate dehydrogenase coupled assay (Creighton & Yanofsky, 1970). Absorbance changes were measured at 37 °C in 1-cm quartz cuvettes using a Shimadzu UV-2100 spectrophotometer. One unit (YU) of activity is defined as the conversion of 0.1 μ mol of substrate to product in 20 min at 37 °C.

SDS-Polyacrylamide Gel Electrophoresis. SDS-PAGE was performed on 15% acrylamide gels using myosin, *E. coli* β -galactosidase, rabbit muscle phosphorylase b, bovine serum albumin, hen egg white ovalbumin, bovine carbonic anhydrase, soybean trypsin inhibitor, and hen egg white lysozyme as molecular weight markers (Laemmli, 1970). Gels were stained with Coomassie blue and scanned with an MPS 940.800 densitometer (Vitatron, Rösraht).

Gel Filtration. Molecular sieve chromatography was performed with a Sephacryl S-200 HR column (80 cm \times 1 cm) equilibrated with buffer A. Sample volumes were between 1 and 2 mL, and flow rates varied between 5 and 10 mL/h. In order to determine complex formation of nicked β_2 with α -subunits according to Hummel and Dryer (1962), a Sephadex G 75 SF column (80 cm \times 1 cm) was equilibrated with buffer A (or buffer B) each containing 23×10^{-6} M α -subunits. One milliliter E-296-nicked β_2 (23×10^{-6} M β -chains) plus α -subunits (23×10^{-6} M) in the presence or absence of pyridoxal 5'-phosphate was applied to the column and eluted with equilibration buffer. Dilution of E-296-nicked β_2 during chromatography leads to a final concentration of about 5×10^{-6} M β -chains and thus to an approximately 5-fold excess of α -subunits over β_2 .

N-Terminal Amino Acid Analysis. Purified and freeze-dried F1a, F1b, and F2 fragments, respectively, were dissolved in 50% acetic acid, and their N-terminal sequences were determined on a Model 440 gas-phase sequencer (Applied Biosystems, Weiterstadt).

^{31}P NMR Spectroscopy. Fourier transform ^{31}P NMR spectra were recorded at 72.86 MHz on a Bruker WH-180 wide-bore superconducting spectrometer according to Schnackerz and Bartholmes (1983).

Analytical Ultracentrifugation. Ultracentrifuge analysis made use of sedimentation velocity experiments in an analytical ultracentrifuge (Beckman, Model E, Palo Alto) equipped with a high-sensitivity ultraviolet-scanning system. Two mixtures of α (2×10^{-5} M) + E-296-nicked holo- β_2 (13.5×10^{-6} M β -chains) and α (2×10^{-5} M) + E-296-nicked apo- β_2 (13.5×10^{-6} M β -chains), respectively, were prepared. The solutions were centrifuged at 44 000 rpm and 22 °C in 30-mm double-sector cells; scans were taken at 280 nm in 16-min intervals.

Circular Dichroism Measurements. Binding of pyridoxal 5'-phosphate to K-87 in the active site of E-296-nicked apo- β_2 was monitored in the visible region by induced circular dichroism in the cofactor on a Jasco J-500 A spectropolarimeter using 1-mm cells. Bound PLP was calculated from the increase of mean residue ellipticity at 415 nm (Miles & Moriguchi, 1977; Balk et al., 1981).

RESULTS

Limited Proteolysis of the β_2 -Subunit by Endoproteinase Glu-C. As can be seen in Figure 3, digestion of the holo-

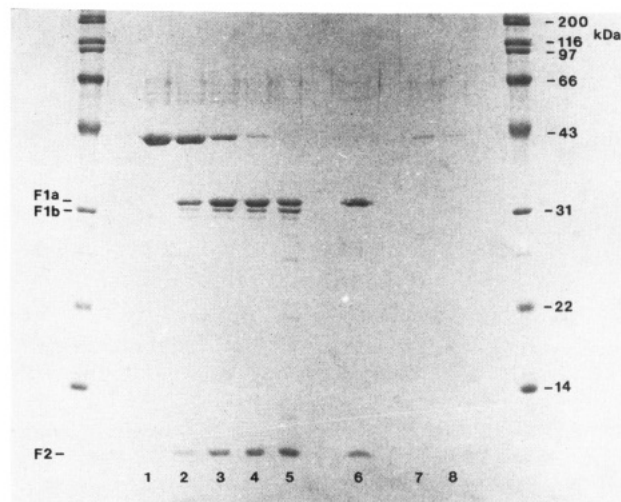


FIGURE 3: SDS-PAGE of fragments produced by proteolysis of β_2 . Lanes 1–5, 0, 2.5, 10, 21, and 60 min of digestion, respectively. Lane 6, F1a/F2 species after anion-exchange FPLC. Lanes 7 and 8, native β_2 -subunit diluted 1:20 and 1:50, respectively.

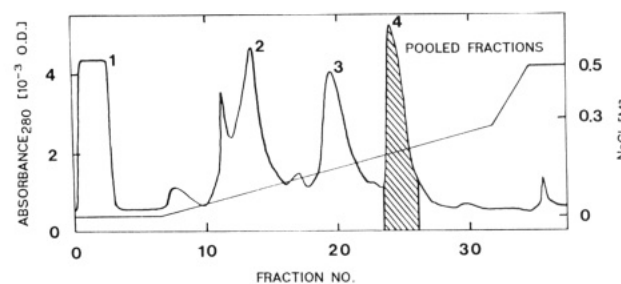


FIGURE 4: Elution profile of Glu-C-nicked β_2 after Mono-Q FPLC. Peaks 1, 2, 3, and 4 contained α_2 -macroglobulin/endoproteinase Glu-C complexes, F1b/F2 chains, F1a/F1b/F2 chains, and F1a/F2 chains, respectively.

β_2 -subunit using endoproteinase Glu-C yields a mixture of one light (F2) and two heavy (F1a and F1b) fragments. Whereas the F2 peptide remains stable, F1a is further converted into F1b during prolonged incubation time (lanes 1–5). α_2 -Macroglobulin was found to be an effective inhibitor for V8 protease (Barrett, 1981) and was used for all preparations of E-296-nicked β_2 after the proteolysis step. Proteolysis was completely inhibited for more than 2 weeks after addition of the inhibitor to the digestion mixture (data not shown). By use of anion-exchange FPLC, F1a/F2 species could be purified to homogeneity (lane 6). A typical elution profile after Mono-Q FPLC can be seen in Figure 4. F1b/F2 species were found in the second, F1a/F1b and F2 chains in the third, and F1a/F2 species in the last peak. Endoproteinase Glu-C complexed with α_2 -macroglobulin did not bind to the column and eluted with the first peak during sample application.

Location of the Cleavage Sites. In order to define the sites of proteolysis, F1a, F1b, and F2 were separately purified to homogeneity. N-Terminal sequence analysis of F2 by Edman degradation led to a partial sequence of S-Y-S-I-S-A starting beyond the C-terminal side of E-296 whereas the N-terminal sequence of both F1a and F1b showed T-T-L-L-N-P which represents the N-terminal partial sequence of the native β_2 -subunit. Molecular weight determination via SDS-PAGE yielded 32 000, 31 000, and 11 000, respectively, for F1a, F1b, and F2. Assuming the sites of proteolytic cleavage of the β -chain to be at E-290 and E-296 and calculating the expected molecular weights of the resulting fragments by using the known amino acid sequence led to 31849.0 for F1a, 31177.3 for F1b, and 11021.7 for F2. In Figure 2, the positions of all

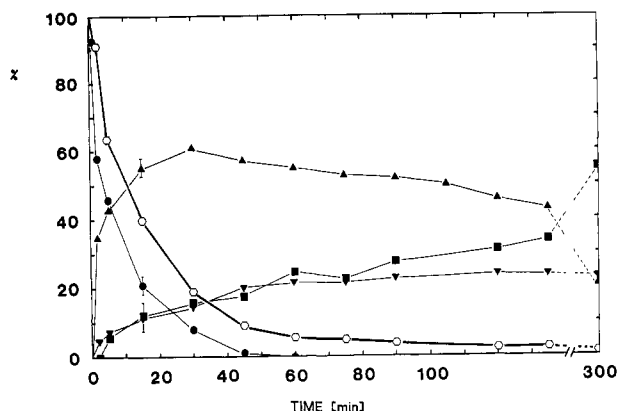


FIGURE 5: Time course of proteolysis of holo- β_2 tryptophan synthase as followed via SDS-PAGE: (●) native β_2 ; (▲) F1a; (■) F1b; (▼) F2; and (○) enzymatic activity measured in the presence of excess α -chains.

Table I: Activities of α , β_2 , and E-296-Nicked β_2 in the Associated and the Isolated State, Respectively^a

reaction	activity (YU)				
	β_2	$\beta_2 + \alpha$	β_2'	$\beta_2' + \alpha$	α
1		1200		108	0.6
2	70	2560	1.6	250	

^a Reaction 1 was measured with a 3-fold molar excess of α -subunits whereas reaction 2 was determined by using a 1.5-fold molar excess of β -chains.

sites of proteolytic cleavage known so far are given.

When the native α_2 /holo- β_2 complex was treated with endoproteinase Glu-C, no proteolysis was detectable.

Kinetic Measurements. The kinetics of deactivation of holo- β_2 during proteolysis are shown in Figure 5. At the point of time where no more native β_2 was detected on SDS gels, residual enzymatic activity was found. As a control for the sensitivity of detection of residual intact β -chains, diluted β_2 -subunits corresponding to 5% or 2% of the original concentration were present on the gel (Figure 2, lanes 7 and 8). Specific activities of purified E-296-nicked β_2 were measured in the presence and absence of α -subunits (Table I). E-296-nicked β_2 shows an activity of 1.6 YU, which is 2.3% of the value found for the native protein when tested in the absence of α -subunits. Adding α to the test mixture leads to 250 YU, which is 10% as compared to the native $\alpha_2\beta_2$ complex. Thus, the catalytic efficiency of nicked β_2 can be enhanced by a factor of 156 when α is added. Furthermore, the activity of α -subunits increases 180-fold when E-296-nicked β_2 is added (Table I).

Binding of α -Subunits to E-296-Nicked β_2 . The ability of E-296-nicked β_2 to bind α -subunits was further demonstrated by gel filtration, ³¹P NMR, and sedimentation velocity experiments.

For conventional molecular sieve chromatography, E-296-nicked β_2 was added to α -subunits (molar ratio of α - to β -chains, 1:1), and 1 mL of the mixture was applied to a Sephacryl S-200 HR column (80 × 1 cm). Subsequent SDS-PAGE analysis of the resulting fractions revealed only weak interaction of the corresponding subunits, leading to an elution profile with insufficient separation of the different species.

Interaction was more clearly demonstrated by applying the technique described by Hummel and Dryer (1962) (Figure 6). At the predicted elution volume of α -subunits, a trough was observed when equimolar amounts of α -subunits and E-296-nicked holo- β_2 were analyzed by molecular sieve chromatography using Sephadex G 75 SF saturated with

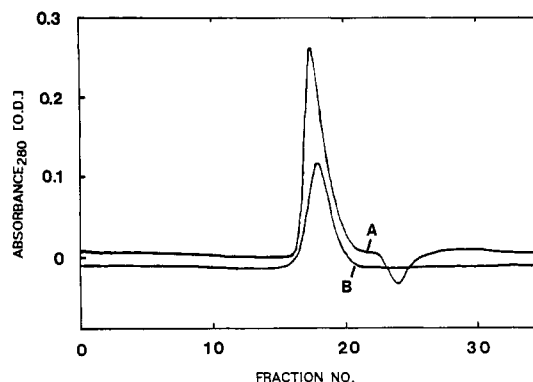


FIGURE 6: Sephadex G 75 SF gel chromatography of modified tryptophan synthase according to Hummel and Dryer (1962). One milliliter of 23×10^{-6} M E-296-nicked holo- β -chains plus 23×10^{-6} M α (profile A) or 1 mL of 23×10^{-6} M E-296-nicked apo- β -chains plus 23×10^{-6} M α (profile B) was applied to a column (80 cm × 1 cm), equilibrated, and eluted with buffer A (for holoenzyme) or buffer B (for apoenzyme) containing 23×10^{-6} M α -subunits.

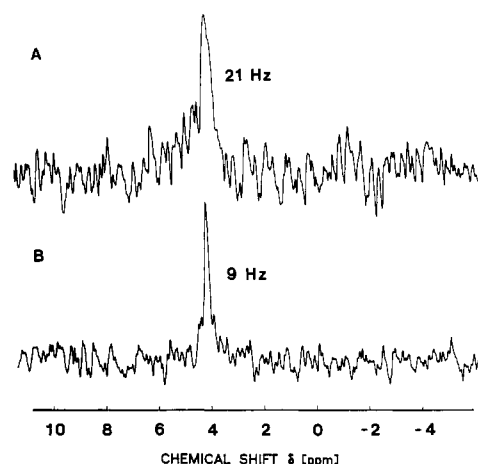


FIGURE 7: ³¹P NMR spectra of pyridoxal 5'-phosphate bound to E-296-nicked β_2 (A) and the α_2 /E-296-nicked β_2 complex (B) at pH 7.3. The chemical shifts are relative to external 85% phosphoric acid. The line widths are 21 Hz (A) and 9 Hz (B).

α -subunits. The area within the trough corresponds to about 33% stoichiometric binding of α -subunits as calculated by using the linear extinction coefficients of native α and β (Hathaway, 1972; Adachi & Miles, 1974). In the absence of pyridoxal 5'-phosphate, the corresponding elution profile included no detectable trough.

In addition, the ³¹P NMR spectrum of E-296-nicked holo- β_2 showed significant band narrowing with line widths of 21 Hz for the dimeric derivative and 9 Hz respectively in the presence of α -subunits (Figure 7), clearly indicating an interaction.

When a 1.5-fold excess of α -subunits mixed with E-296-nicked holo- β_2 protein was tested in sedimentation velocity experiments, two species were detected (Figure 8): the first one with a sedimentation velocity constant of 2.5 S, corresponding to free α -subunits, and the second one with an *s* value of 5.2 S, which is significantly higher than 4.9 S, as found for isolated β_2 (Goldberg et al., 1966; Creighton & Yanofsky, 1966; Bartholmes & Teuscher, 1979). The sedimentation profile of E-296-nicked apo- $\beta_2 + \alpha$ revealed no species sedimenting faster than 4.9 S.

Binding of Pyridoxal 5'-Phosphate to E-296-Nicked β_2 . The binding characteristics of pyridoxal 5'-phosphate to E-296-nicked apoenzyme were determined by CD titration at 415 nm. Figure 9 shows the obtained results in a Scatchard plot which clearly demonstrates that pyridoxal 5'-phosphate binding is noncooperative with $K_d = 5 \times 10^{-6}$ M.

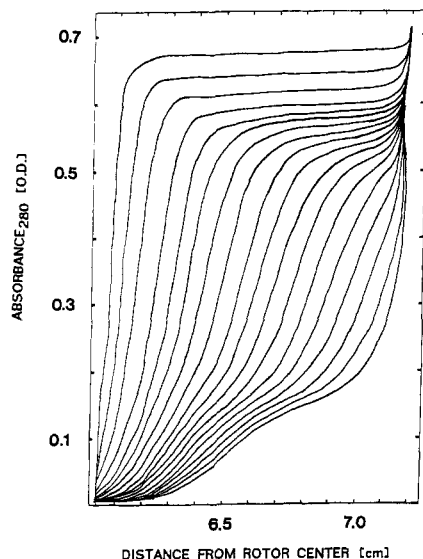


FIGURE 8: Absorbance profiles of a typical sedimentation velocity experiment with E-296-nicked holo- β_2 and α -subunits at 280 nm. The time interval between two scans was 16 min. Initial concentrations were 13.5×10^{-6} M E-296-nicked holo- β -chains plus 2×10^{-5} M α -subunits.

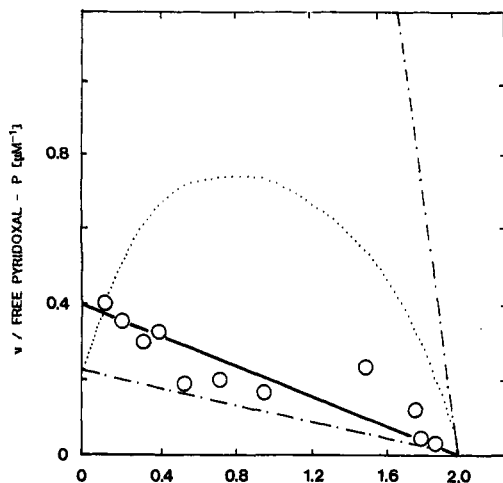


FIGURE 9: Binding of pyridoxal 5'-phosphate to proteolytically modified tryptophan synthase measured via CD titration at 415 nm. (O) 13.5×10^{-6} M E-296-nicked apo- β -chains; (—) linear least-squares fit to $v/[cofactor_{free}] = (n - \nu)/K_d$, yielding $K_d = 5 \times 10^{-6}$ M and $n = 2$. This is compared to cooperative cofactor binding to 12.25×10^{-6} M native apo- β -chains. (---) Fitted data taken from Bartholmes et al. (1976). (· · ·) Slopes corresponding to $K_{d1} = 8.7 \times 10^{-6}$ M and $K_{d2} = 2.3 \times 10^{-7}$ M.

DISCUSSION

The interaction of α - and β_2 -subunits in the $\alpha_2\beta_2$ bienzyme complex of tryptophan synthase represents an example for the sensitive control of both the structure and catalytic efficiency of a complex enzyme due to the mutual influence of its chemically different constituents (Faeder & Hammes, 1970; Kirschner et al., 1975; Weisheit & Kirschner, 1976). In addition, the structural stability of the enzyme is significantly influenced by the coenzyme of the β_2 -subunit, pyridoxal 5'-phosphate (Bartholmes et al., 1976; Miles & Moriguchi, 1977; Bartholmes & Teuscher, 1979; Wiesinger et al., 1979; Seifert et al., 1984, 1985), which forms an internal aldimine with a lysyl residue (K-87) in the active center of the β -chain. Moreover, the delicate interplay of distinct structural domains within the tertiary structure of both subunits as a consequence of the binding of small ligands adds another aspect to the

interactions operative in maintaining the tertiary structure as well as the fine regulation of the enzymatic activity (Högberg-Raibaud & Goldberg, 1977b; Lane & Kirschner, 1981; Bartholmes, 1984). Perturbation of the native tension of the structure by limited proteolysis at readily susceptible cleavage sites (which in general are considered as poorly structured regions between domains) results in remarkable obstruction of relevant functional parameters as far as complete inactivation of the enzyme (Högberg-Raibaud & Goldberg, 1977b; Miles & Higgins, 1978; Bartholmes et al., 1979; Higgins et al., 1979).

In the present experiments, the influence of the location of limited proteolytic cleavage of the β -chain on structure and catalytic efficiency of the bienzyme complex has been investigated. From earlier reports, it is well established that upon action of trypsin as well as endoproteinase Lys-C the peptide between K-272 and K-283 (Goldberg-loop) can be removed. In this enzymatically inactive product, the two resulting fragments are still tightly associated. Substrates and the coenzyme pyridoxal 5'-phosphate are bound with significant residual affinity, but no complex formation with the corresponding α -chains could be detected (Högberg-Raibaud & Goldberg, 1977a,b). More recently, it has become clearly evident from the 2.5-Å X-ray structure of the closely homologous *S. typhimurium* enzyme that the Goldberg-loop constitutes only about 20% of an elongated 53-residue stretch within the C-terminal domain of the β -chain (Hyde et al., 1988). Using endoproteinase Glu-C from *S. aureus* V8 strain, it was possible to proteolyze the β -chain within this larger peptide, but far outside the Goldberg-loop. Three Glu-C cleavage sites exist in this region: E-290, E-295, and E-296. N-Terminal sequencing of F1a, F1b, and F2 and SDS-PAGE data can be taken as an evidence that the two sites of proteolytic cleavage are at E-290 and E-296. Although an average deviation in molecular weight determination using SDS-PAGE of 5% cannot be excluded, the molecular weight difference with respect to the next possible cleavage site exceeds this expected error (8.5% for the second possible cleavage at E-266). The obtained results do not allow the decision, whether the C-terminal amino acid residue in F1a, i.e. E-296 is removed or not. The modified β -chains containing only the larger N-terminal fragment could be purified from the incubation mixture after inhibition of endoproteinase Glu-C with α_2 -macroglobulin. This protein is dimeric and still binds two molecules of pyridoxal 5'-phosphate, however, with hyperbolic characteristics yielding an apparent microscopic dissociation constant K_d of 5×10^{-6} M. This result is similar to the dissociation constant of coenzyme binding to the β -chain lacking the Goldberg-loop ($K_d = 3.4 \times 10^{-6}$ M, Balk, 1981), which clearly indicates that the integrity of the β -chain is a necessary prerequisite for the conformational change as taking place upon cooperative pyridoxal 5'-phosphate binding to the native enzyme with $K_{d1} = 8.7 \times 10^{-6}$ M for the low-affinity T state and $K_{d2} = 2.3 \times 10^{-7}$ M for the high-affinity R state (Bartholmes et al., 1980; Balk et al., 1981b).

Incubation of the native $\alpha_2\beta_2$ complex with endoproteinase Glu-C does not lead to proteolysis. In a similar way, the latent trypsinolytic cleavage sites of β_2 are protected upon complex formation with α (Higgins et al., 1979). The isolated β_2 derivatives show significant differences in their ability to interact with α -subunits, thus indicating that the Goldberg-loop is more directly involved in subunit contact than the E-296 site. On the other hand, it is well-known that partial trypsinolysis of the α -subunit only leads to the above-mentioned, enzymatically active modification product with one cleavage

site at R-188 (Higgins et al., 1979) when trypsin acts on the $\alpha_2\beta_2$ complex. Obviously, the corresponding β -chains are able to effectively shield other cleavage sites for trypsin in the intersubunit contact region which are readily susceptible within the isolated α -subunit.

Protein complexes which only weakly interact often show enhanced dissociation upon conventional molecular sieve chromatography, thus pointing to apparent dissociation constants higher than the true values. In fact, considerable amounts of free α -subunits were detected in a trailing edge of the elution profile on a Sephacryl S-200 HR column loaded with α_2 /E-296-nicked holo- β_2 , indicating incomplete complex formation. Therefore, we applied the method introduced by Hummel and Dryer (1962) which allows a more reliable estimation of complex stability, since the heavier complex migrates through a constant concentration of α -subunits included in the elution buffer. As shown by this technique, E-296-nicked apo- β_2 does not significantly interact with α -subunits during chromatography. At similar protein concentrations, E-296-nicked holo- β_2 unambiguously forms a complex with α -subunits as may be quantified from the observed trough in the elution profile. Since no exact titration data are yet available, it remains an open mechanistic question whether the calculated saturation of 33% can be taken as indication for a dissociation constant of approximately 2×10^{-5} M for α_2 /E-296-nicked holo- β_2 . An intermediate α /E-296-nicked holo- β_2 complex and hence negative cooperativity of complex formation may be discussed as well. Recently, negative cooperativity for self-assembly via an $\alpha\beta_2$ intermediate has been convincingly demonstrated for the native α_2 /holo- β_2 complex by the elegant approach of Lane et al. (1984), leading to $K_d = 0.26 \times 10^{-9}$ M for the $\alpha\beta_2$ species.

It can be regarded as additional evidence for subunit interaction that the internal mobility of the cofactor bound to E-296-nicked holo- β_2 is considerably enhanced upon interaction with α -subunits as demonstrated by significant line narrowing in the corresponding ^{31}P NMR spectra. This finding corroborates earlier results from studies with intact holo- β_2 -subunits (Schnackerz, 1984; Bartholmes et al., 1987).

After all, we tried to trace complex formation between α -subunits and the modified protein by an ultracentrifuge experiment originally invented in order to quantify the interaction between proteins and nucleic acids (Revzin & von Hippel, 1977; Revzin & Woychik, 1981). E-296-nicked holo- β_2 is allowed to sediment in a slight molar excess of α -subunits. The resulting sedimentation pattern again clearly indicates a real association-dissociation equilibrium in the presence of coenzyme. No interaction can be detected in the absence of pyridoxal 5'-phosphate. Quantitative determination of a complex dissociation constant is possible with an iterative algorithm (unpublished results).

Although the primary sequence is interrupted in the isolated E-296-nicked holo- β_2 -subunit, residual enzymatic activity can be detected. The observed activity is certainly not due to trace amounts of unproteolyzed enzyme since intact β_2 -subunits diluted to 2% and 5%, respectively, of the original concentration can be easily detected on SDS-PAGE (Figure 2, lanes 7 and 8). On the other hand, the trypsinolyzed β_2 -dimer is completely inactive. Hence, we may conclude that in E-296-nicked holo- β_2 sufficient structural stabilization is provided by neighboring elements of secondary structure which are absent in a product lacking the Goldberg-loop. Additionally, the described enhancement of specific activity of both subunits upon complex formation provides clear evidence that quasi-physiological conformational changes are mutually induced

which in the case of E-296-nicked holo- β_2 do not strictly depend on an intact primary structure. Under the conditions of the enzymatic test, a stabilizing effect of the substrates on the association between α and E-296-nicked β_2 is likely to occur. Indeed, though a reliable estimate of complex formation cannot be made, gel chromatography suggests an apparent dissociation constant of approximately 2×10^{-5} M, a value much higher than the subunit concentration in the assay. Thus, the observed mutual activation might not necessarily be regarded as a maximum value. Future work will deal with equilibrium and kinetic experiments in order to elucidate the mechanism of interaction of both α -subunits with E-296-nicked holo- β_2 .

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Temperature and pH Dependence of the Self-Association of Human Spectrin[†]

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ABSTRACT: The self-association of human spectrin between 21 and 35 °C and between pH 6.5 and 9.5 has been studied at sedimentation equilibrium. For a given set of solution conditions between pH 6.5 and 8.5, coincidence of Ω function plots as a function of total spectrin concentration (0–2 g/L) indicated that equilibrium was attained and that no significant concentration of solute was incapable of participating in the self-association reaction. Above pH 8.5, however, irreversible aggregation occurred, inferred from a failure of overlap in the Ω function and molecular weight distributions. The behavior of spectrin can best be described by a cooperative isodesmic model, in which the protomer for association is the heterodimer and for which K_{12} is between 10^6 and 10^7 M⁻¹ (depending on pH and temperature) and all other K are approximately 10^6 M⁻¹. The returned values of the second virial coefficient for this model fall within the range calculated from the charge and Stokes radius of spectrin. Association appears to be favored slightly by decreased temperature and by decreased pH. The pH dependence resides only in K_{12} and is consistent with the presence of a single group, possibly histidine, displaying a slightly higher pK_a value in the tetramer than in the dimer. The association reaction appears to be driven by the loss of enthalpy associated with release of strain in the heterodimer. The association sites appear to be conserved in the association reactions, consistent with the images from electron microscopy. Within the precision of the data, the loss of rotational and conformational entropy on closing the oligomers from their open-chain forms is independent of the size of the oligomer.

Spectrin is the major protein of the erythrocyte membrane cytoskeleton that lines the cytoplasmic face of the red-cell membrane (Palek & Lux, 1983). The basic structural unit of spectrin is the heterodimer: a long, wormlike molecule consisting of two different polypeptide chains loosely wound around each other (Shotton et al., 1979). Spectrin is capable of self-associating through the sequential addition of heterodimers to form tetramers (Ralston, 1978; Shotton et al., 1979) and higher oligomers (Morrow & Marchesi, 1981; Morrow et al., 1981; Morris & Ralston, 1984; Liu et al., 1984).

In previous sedimentation equilibrium studies with spectrin at pH 7.5 and 30 °C (Morris & Ralston, 1989), it was not possible to decide unambiguously between two plausible models for indefinite self-association. In the “cooperative isodesmic” model (SEK III; Tang et al., 1977), the equilibrium constant in the molar scale for dimerization of the protomer, K_{12} , has a value different from the equilibrium constant in the molar scale that describes all subsequent additions of protomer to preexisting oligomers, K_{iso} . In the “attenuated indefinite” model (AK I; Adams et al., 1978), the sequential equilibrium constants are related to an “intrinsic constant”, K , by the relationship $K_{i-1,i} = K/i$.

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