Journal of Chromatography, 115 (1975) 205–212

CHROM. 8461

PURIFICATION OF *ESCHERICHIA COLI* B-SPECIFIC *p*-AMINOBENZOATE "PICK-UP" PROTEIN TO HOMOGENEITY BY AFFINITY CHROMATOGRAPHY

BELA L. TOTH-MARTINEZ, ZOLTÁN DINYA and FERENC HERNÁDI

Department of Pharmacobiochemistry, Institute of Pharmacology, Medical University of Debrecen, 4012 Debrecen (Hungary)

(First received March 25th, 1975; revised manuscript received May 28th, 1975)

SUMMARY

Of the satellite fractions of *Escherichia coli* B dihydrofolate synthetases, a non-enzymic protein that is specifically able to bind *p*-aminobenzoate and sulphonamides has been purified 6000-fold by *p*-aminobenzoylcellulose affinity chromatography. The protein was named *p*-aminobenzoate "pick-up" protein according to its function, *i.e.*, to bring *p*-aminobenzoate into reaction with L-glutamate and pteridine during dihydrofolate biosynthesis. About 4 mg of pure protein (0.532% recovery, calculated from the total *p*-aminobenzoate binding capacity of the unfractionated supernatant separated from the crude bacterium plasma) can be obtained from 500 g of harvested cells. The product is homogeneous in polyacrylamide gel electrophoresis both in the absence and presence of sodium dodecyl sulphate, and has a molecular weight of 15,000 daltons ± 5% as measured by sodium dodecyl sulphate gel electrophoresis and Sephadex G-75 gel column chromatography. *p*-Aminobenzoate and sulphonamide ligand binding studies showed a single binding site per *p*-aminobenzoate pick-up protein molecule. K_D values for *p*-aminobenzoate and some sulphonamides as well as for L-glutamate, L-γ-glutamyl oligopeptides, some pteridines and folate antagonists are also presented in order to illustrate the specificity of the receptor protein.

INTRODUCTION

Enzymes of the tetrahydrofolate (THFA) assembling system of *E. coli* have been found[1–4] to be able to associate *in vitro* and form partial heterologous protein associates with each other or with some polypeptides that have not so far been identified but seem to be impurities of these enzyme preparations. From partially purified hydroxymethylpteridine pyrophosphokinase (HDPK), dihydropteroate synthetase (DHPAS)[2] and dihydrofolate synthetase (DHFAS)[4] enzyme preparations, several satellite protein fractions were separated[5], including a *p*-aminobenzoate (pABA) and sulphonamide (SA) binding fraction of non-enzyme character, which has been termed as *p*-aminobenzoate pick-up protein (pABApup). Enzyme kinetic investigations revealed that pABApup is a subunit-like component of the DHPAS and the DHFAS[2,6,7]. It is also able to associate with the *E. coli* pABA:ATP adenylate kinase (pABA:ATP

AK) and the *p*-aminobenzoylglutamate synthetase (pABGAS)[8], forming together a ternary protein complex system. *In vitro* biosynthesis of *p*-aminobenzoylglutamate (pABGA) is accomplished by the complex.

As new possibilities emerged for the participation of pABA in the biosynthesis of THFA via dihydrofolate (DHFA), based on the association capabilities of the enzymes involved in the process[5,9-12], research to produce the components of the individual enzymes in an essentially pure and native form has become more important.

We have been interested in the mechanism of the action of SAs and Trimetoprim (TMP) for several years as we hoped to be able to understand their potentiated synergy by learning more about the enzymes themselves upon which the SAs and TMP act, and similarly about the multienzyme complex (MEC) nature of the THFA assembling bacterial systems, and finally about the true nature of the SA and TMP resistance, which might involve genetic alterations, especially in the primary structure of the pABApup component in the THFA–MEC of THFA biosynthesizing bacteria.

A method for the preparation of *E. coli* B pABApup in homogeneous form for pharmacobiochemical studies is described in this paper.

MATERIALS AND METHODS

Cellulose powder for chromatographic purposes was obtained from Whatman (Maidstone, Great Britain). All chemicals used for *p*-aminobenzoylcellulose (pAB-cellulose) preparation were purchased from Merck (Darmstadt, G.F.R.) and imported by Reanal (Budapest, Hungary). Chemicals used during the preparation of the enzymes, enzyme activity determinations and inhibition studies with SAs and TMP were supplied by Merck Boehringer (Mannheim, G.F.R.) and The Radiochemical Centre (Amersham, Great Britain). DHFA, dihydropteroate (DHPA) and dihydropteridine (Pter) were freshly prepared according to Zakrzewski and Sansone[13]. Pteridine pyrophosphate (PterPP) was prepared as described by Shiota *et al.*[14]. Phosphorus was determined according to Hesse and Geller[15]. Total protein was measured with the biuret method[16,17] and the purified enzyme–protein was measured spectrophotometrically[18]. A method for pABA and SA determination has been published elsewhere[19]. Assays for pABA binding by equilibrium dialysis studies were made according to Breslow and Walter[20] as modified by ourselves[12]. *E. coli* B was fermented in a synthetic medium as described by Kornberg *et al.*[21]. HDPK and DHPAS were prepared according to Richey and Brown[3] and by our method[5,12] and DHFAS I and II were made either according to Brown[4] or by our own method[5,12]. Purification of the enzymes of pABGA synthesis was carried out according to our enzyme preparation method[5,12]. HDPK and DHPAS[3], DHFAS[4], pABGAS[8] and dihydrofolate reductase (DHFR)[22] enzymes were assayed in different column chromatography fractions as shown in Fig. 1. Polyacrylamide gel electrophoresis and gel chromatography with Sephadex G-75 superfine was performed in a routine manner.

The preparation of pAB-cellulose was carried out as follows. Dry cellulose powder (100 g) was suspended in 1 l of dry pyridine in the cold in a three-necked reaction flask fitted with reflux and mechanical stirring facilities. Four moles of *p*-nitrobenzoyl chloride (pNBC) per mole of glucose were slowly introduced into the suspension with vigorous stirring and cooling. The reaction mixture was then allowed to warm to room temperature under control and was further stirred for 10 h. The reac-

tion was completed in an ultrathermostat in two stages: (a) 10 h stirring at 40° and (b) 10 h stirring at 60°. After completion of the reaction, the mixture was allowed to cool to room temperature and was then poured into 5 l of chilled 10% (w/v) sodium hydrogen carbonate solution. The gum was dissolved in 3 l of chloroform. The aqueous layer was extracted three times with 1-l portions of chloroform. The combined chloroform extracts were washed with dilute sulphuric acid (5%, w/v), 10% sodium hydrogen carbonate solution and distilled water, and finally dried over calcium sulphate. The organic fraction was evaporated *in vacuo*, giving a yield of about 150 g of pNB-cellulose. The crude product was then re-suspended in portions of 15 g in 500 ml of ethyl acetate and 5 ml of glacial acetic acid were added to it. Catalytic hydrogenation was then performed following the addition of 1.5 g of a 10% palladium–carbon catalyst and equilibration at room temperature. After completion of hydrogenation in about 5 h, the solvent was evaporated *in vacuo*. The residue was re-suspended in distilled water and sedimented to separate the light catalyst, which floated. Four or five repetitions of the procedure proved to be satisfactory. Elimination of some coloured material with 0.05 *M* sodium hydroxide solution and 0.05 *M* hydrochloric acid was desirable. Further purification is achieved during equilibration of the material with buffers of different pHs and salt concentrations, giving a yield of 130 g of pAB-cellulose.

The procedure for the purification of pABApup includes the preparation of a crude extract of *E. coli* B and treatment with RNase according to Brown[22], involving treatment with dry Sephadex G-5 gel of the diluted supernatant of 20–25 mg/ml of protein (3 g per 100 ml of extract) followed by low-speed centrifugation for the removal of the gel. This step cannot be omitted without the risk of losing pABApup because Sephadex G-5 removes substances that can interfere in the binding of pABApup to the affinity material (excess of salts, small organic molecules, etc.). The detailed purification procedure is as follows. Operations were performed at 5–10°. pAB-cellulose (50 g) was equilibrated with 0.05 *M* potassium phosphate (pH 7.4) containing 0.05 *M* EDTA. The slurry was sedimented by centrifugation, transferred into the pre-treated extract and the suspension was stirred gently for 3–5 h. The solids were allowed to settle and transferred into a column with some of the supernatant. The column was then washed with the above buffer until the absorbance at 280 nm was about 0.3 (A_{280}^{1cm}). The flow-rate was 1 ml/min. The column was further washed with 0.05 *M* potassium phosphate (pH 6.8) containing 0.1 *M* potassium chloride and 0.05 *M* EDTA until the absorbance became less than 0.05. Then a 0.3–0.5 *M* potassium chloride gradient in a 0.05 *M* potassium phosphate buffer (pH 8) was applied to remove pABApup. This technique was more convenient than applying 0.5 m*M* pABA for removal of pABApup from the column in the pH 8 buffer. pABApup was concentrated to a few millilitres by dialysis against solid sucrose or by passing it through a 20 × 2 cm Sephadex G-15 column equilibrated with 0.05 *M* potassium phosphate buffer (pH 7.5) when pABA was used as eluting agent. The affinity material was regenerated by washing overnight with 0.05 *M* potassium phosphate buffer (pH 8.5) containing 1 *M* potassium chloride. Finally, equilibration with the starting buffer, containing 0.02% of sodium azide, made it ready for use. The results of the purification steps are outlined in Table I.

The affinity material has been re-used several times with no significant change in its performance.

TABLE I

PURIFICATION OF pABApup BY AFFINITY CHROMATOGRAPHY

Purification step	*Volume (ml)*	*Total protein (mg)*	*nM of pABA (bound) per mg of protein**
Crude extract	1,000	23,970	2.08
Sephadex G-5-treated extract (diluted)	1,800	19,700	1.82
Affinity chromatography	35	3.9	66

* The great difference in the binding of pABA before and after affinity chromatography is mainly attributed to the non-specific binding of the ligand to bacterial proteins other than pABApup. A certain amount of apo-pABApup may be lost during the first two purification steps, and further loss of the pABApup–pABA complex during the elution step with pABA is also considered.

RESULTS AND DISCUSSION

pAB-cellulose affinity chromatography was first used to purify DHFAS I and II[5] prepared according to Brown[4] (Fig. 1), and also for the subunit analysis of the pABGAS system[8]. Recently it was used for the comparative subunit analysis of DHPAS and DHFAS enzymes[7,24]. Identification of pABApup from all of these enzyme preparations has thrown light on its central role in THFA biosynthesis, namely that this is the protein that is responsible for binding and channelling pABA into the correct position for the above purpose, or doing the same with the SA molecule, leading to the lethal synthesis of a false DHFA compound, as was shown by Bock *et al.*[25].

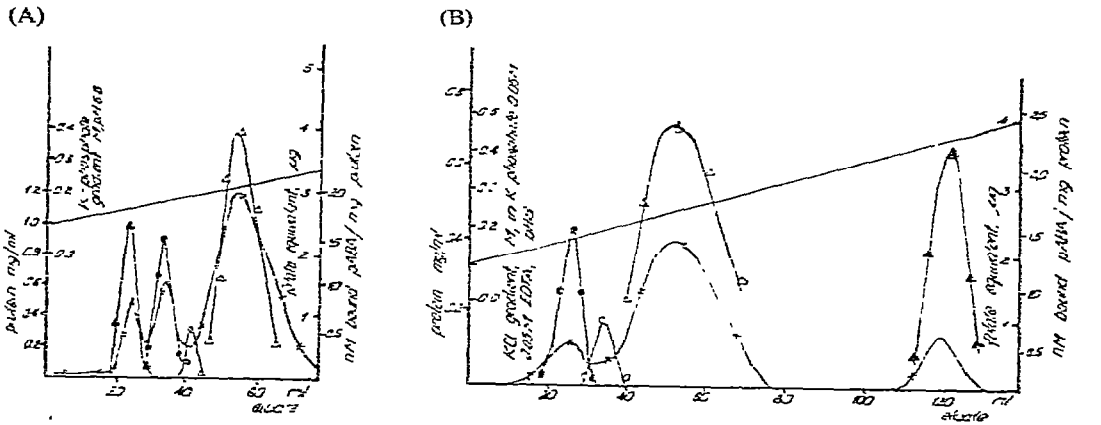

Fig. 1. Re-chromatography of DHFAS I in 4 *M* guanidine hydrochloride + 0.05 *M* EDTA on (A) a DEAE-cellulose column and (B) on a pABA-cellulose column. DHFAS I was prepared according to Brown[4]. ×, Protein eluted from the columns with the respective gradient buffer systems as stated in the diagrams; ▲, pABApup as measured by the pABA binding capacity of the samples in micro equilibrium dialyzer; ●, pABGAS activity values; ○, DHFR activity values; △, DHFAS activity values. Enzyme activity data were expressed in μg folate equivalents. DEAE-cellulose re-chromatography of DHFAS I was performed as follows: about 5 mg of total protein was applied on the column (20 × 1 cm); elution rate, 0.3 ml/min; fraction vol., 2 ml. pABA-cellulose chromatography was carried out with 8 mg of total protein applied on a 30 × 1.5 cm column; elution rate, 0.3 ml/min; fraction vol., 2 ml. All experiments were conducted in a cold room at 5–10°.

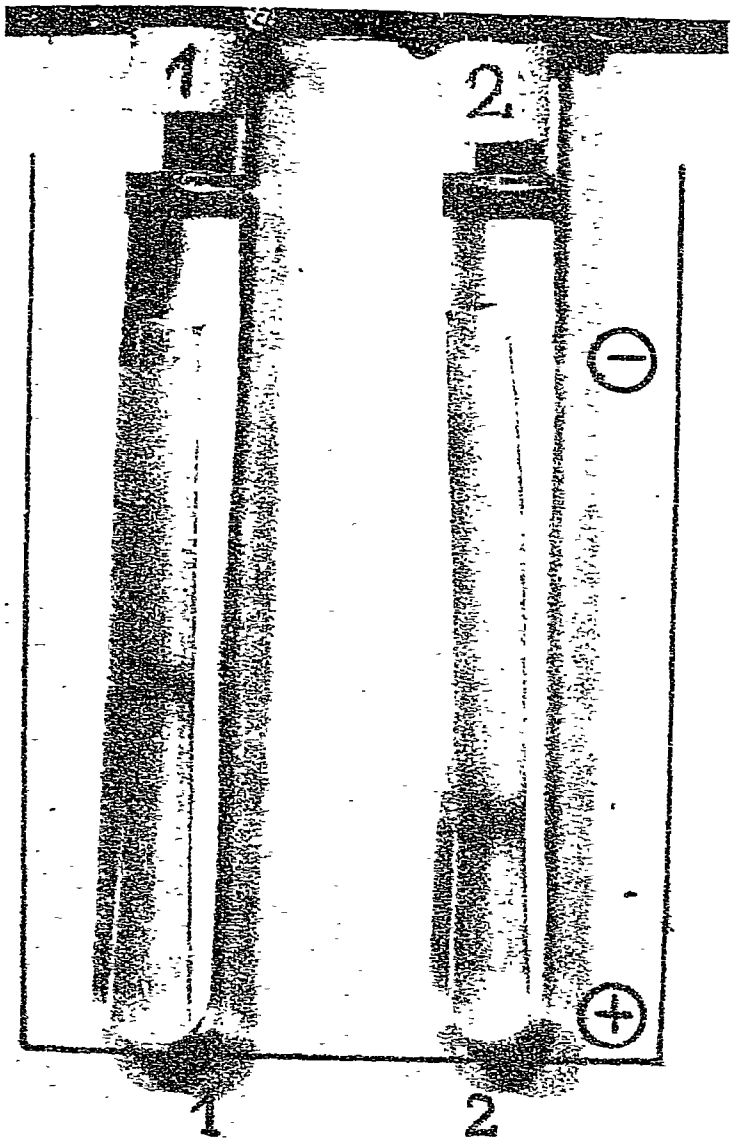

Fig. 2. Analytical gel electrophoresis of pABApup purified by affinity chromatography. Gel 1: 25 μg of protein was electrophoresed according to Davis[26] with the exception that both separating and stacking gels contained riboflavin and were photopolymerized. Electrophoresis was carried out at 3.2 mA per gel and was performed in the cold. Gel 2: 25 μg of protein was run in the same manner, except that the sample was pre-treated with SDS and electrophoresed as described by Weber and Osborn [27].

The purity of the preparation was examined by electrophoresis of the native protein on the polyacrylamide system of Davis[26] and in the presence of sodium dodecyl sulphate (SDS) by the method of Weber and Osborn[27]. As shown in Fig. 2, only a single band is observed. The electrophoretic mobility of the SDS-treated protein in the presence of the marker proteins bovine serum albumin, ovalbumin and car-

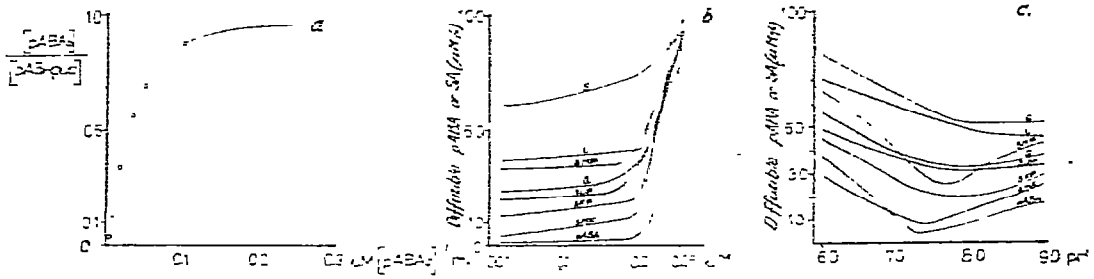

Fig. 3. Stoichometry of binding of pABA and SAs to pABApup. (a) [pABA$_b$], pABA bound to protein (μM); [pABA$_f$], free pABA (μM); [pABApup], total concentration of pABApup (μM). Equilibrium microdialysis was performed in 0.05 M potassium phosphate buffer (pH 7.5) at 20°. pABApup concentration varied between 0.01 μM (0.15 mg/ml) and 0.1 μM (1.5 mg/ml). Total pABA concentration, 0.3 μmole/ml. Dialyzer: total volume, 1.5 ml; inner volume, 0.3 ml. (b) Dependence of ligand binding on its concentration at constant pABApup concentration (0.2 μmole/ml). (c) Dependence of ligand binding on pH at constant pABApup concentration (0.2 μmole/ml). SAs: Sulfamethoxazole (SMX), Sulfaphenylpyrazole (SFP), Superseptyl (SUP), Quinoseptyl (Q), Sulfamethoxypyridazin (SMP), Ultraseptyl (U) and Sulfanylamide (S).

boxypeptidase-A gives a molecular weight of 15,000 daltons ± 5%. The molecular weight of pABApup was also tested by Sephadex G-75 superfine gel chromatography. Calibration of the column was carried out according to Andrews[28]. The results of the ligand binding studies with pABA and several SAs are shown in Fig. 3.

TABLE II

APPARENT DISSOCIATION CONSTANTS (K_D) OF pABApup FOR DIFFERENT SUBSTRATES AND INHIBITORS OF DHFS

Material		K_D (μM)
L-Glutamate		600
L-γ-Glutamyl-L-glutamate		580
L-γ-Glutamyl-L-γ-glutamyl-L-glutamate		580
S	sulphonamide group*	128
U		90
SMP		80
Q		60
SUP		42
SFP		32
SMX		13
pABA		9
7,8-Dihydropteridine		480
7,8-Dihydro-6-(pyrophosphorylhydroxymethyl)pteridine		410
7,8-Dihydropteroic acid		365
7,8-Dihydrofolic acid		300
5,6,7,8-Tetrahydrofolic acid		325
ATP		650
ADP		610
Inorganic pyrophosphate		820
TMP		490
Aminopterin		470
Methotrexate		400

* Abbreviations for the sulphonamide groups as in Fig. 3.

The stoichiometry of these experiments showed that there is one binding site for pABA or SAs per molecule. SAs are also strong eluting agents of pABApup from the affinity material, but they behave differently according to their affinity for the protein. Differences among SAs in their affinity for pABApup also express their differences in inhibiting DHFA synthesis in *in vitro E. coli* B systems[5] (Table II). K_D were measured by monitoring changes in absorption as a function of concentration of ligands at difference spectral maxima[29]:

$$\Delta A^{\lambda max} = \Delta A^{\lambda max}_{max} - K_D \cdot \frac{\Delta A^{\lambda max}}{[L]_f}$$

where $\Delta A^{\lambda max}_{max}$ and $\Delta A^{\lambda max}$ are the actual maximal and measured changes in absorption at λ_{max}, respectively, and $[L]_f$ is the equilibrium concentration of the particular free ligand (L). As the molar excess of the Ls is relatively high, the amounts of Ls bound to bABApup was regarded as negligible in relation to its total amount. The experimental points fall on straight lines, which means that binding of each L to pABApup can be characterized by a single dissociation constant. Reaction mixtures for ligand binding studies have been published elsewhere[5,12]. pABApup was electrophoretically homogeneous. The temperature was 15° (thermostatted) and the buffer system was as used in enzyme activity assays[3,4,8].

REFERENCES

1 T. Shiota, M. N. Disraely and M. P. McCann, *J. Biol. Chem.*, 239 (1964) 2259.
2 P. J. Ortiz and R. D. Hotchkiss, *Biochemistry*, 5 (1966) 67.
3 D. P. Richey and G. M. Brown, *Methods Enzymol.*, 18 (1971) 765.
4 G. M. Brown, *Methods Enzymol.*, 18 (1971) 771.
5 B. L. Toth-Martinez, S. Papp, Z. Dinya and F. Hernádi, *Hung. Pharmacol. Soc. Chemother. Sect., 4th Meeting, Siklós, Hungary, 1974, Detailed Programme*, pp. 67–71 and 72–86.
6 B. L. Toth-Martinez, S. Papp, Z. Dinya and F. Hernádi, *Joint Pharmacobiochem. Meeting of Hung. Chem. Soc. Biochem. Sect. and Hung. Pharmacol. Soc. Pharmacobiochem. Sect., Budapest 1974, Programme*, p. 2.
7 B. L. Toth-Martinez, Z. Dinya and F. J. Hernádi, *Abstr. 6th Int. Congr. Pharmacol., Helsinki 1975*, p. 122.
8 B. L. Toth-Martinez, S. Papp, Z. Dinya and F. Hernádi, *Pharmacol. Meeting Graz. Biochem. Pharmacol. Sect. III/2, 1974, Programme*, p. 30.
9 B. L. Toth-Martinez, S. Papp, Z. Dinya and F. Hernádi, *Abstr. 9th FEBS Meeting, Budapest, 1974*, Abstr. c3b17, p. 341.
10 B. L. Toth-Martinez, S. Papp, Z. Dinya and F. Hernádi, *Abstr. GIM '74 2nd Int. Symp., Sheffield, 1974*, p. 37.
11 B. L. Toth-Martinez, *Conf. on THFA–MEC, Borstel Res. Inst., Borstel, G.F.R., 1974.*
12 B. L. Toth-Martinez, S Papp, Z. Dinya and F. Hernádi, *BioSystems*, 5 (1975)
13 S. F. Zakrzewski and A. M. Sansone, *Methods Enzymol.*, 18 (1971) 726.
14 T. Shiota, M. N. Disraely and M. P. McCann, *Biochem. Biophys. Res. Commun.*, 7 (1962) 194
15 G. Hesse and K. Geller, *Mikrochim. Acta*, (1968) 526.
16 E. Layne, *Methods Enzymol.*, 3 (1957) 450.
17 J. Goa, *Scand. J. Clin. Lab. Invest.*, 5 (1953) 218.
18 E. Layne, *Methods Enzymol.*, 3 (1957) 451.
19 B. L. Toth-Martinez, M. Fodor, I. Békési and Gy. Lehoczky, *Acta Pharm. Hung.*, 44 (1974) 241.
20 E. Breslow and R. Walter, *Mol. Pharmacol.*, 8 (1972) 75.
21 A. Kornberg, S. R. Zimmerman, J. Kornberg and J. Josse, *Proc. Nat. Acad. Sci. U.S.*, 45 (1959) 772.