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N-terminal amino acid sequence of the chromosomal dihydrofolate reductase purified from trimethoprim-resistant *Staphylococcus aureus*

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The existence of two distinct dihydrofolate reductases (DHFR) in highly trimethoprim-resistant clinical isolates has been unequivocally demonstrated. The enzymes have been characterized with regard to the affinity for substrates and sensitivity to inhibitors. The chromosomal, trimethoprim-sensitive DHFR was purified to homogeneity by a new simple two-step procedure. Its N-terminal amino acid sequence, determined up to the first 35 amino acids, showed 69% homology with the *Escherichia coli* DHFR.

Trimethoprim resistance; Dihydrofolate reductase; Amino acid sequence; (*Staphylococcus aureus*)

1. INTRODUCTION

Trimethoprim (Tp)-resistance, which is fairly common in Enterobacteriaceae today, has recently also been found in staphylococci [1–3]. It is frequently associated with other resistances, such as methicillin, gentamicin, erythromycin and others [3,4]. The Tp-resistance gene has been located on plasmids or on the chromosome [3,5]. It mediates a high level of Tp-resistance (MIC $\geq$ 1000 μg/ml) due to the production of a new dihydrofolate reductase (DHFR). The DHFR encoded by plasmid pSK1 was recently partially purified and designated as type S1 [6]. It is a monomer of M_r = 19700 and exhibits reduced sensitivity towards Tp. Neither the origin of the novel DHFR gene, nor the biochemical relationship of the gene product with the chromosomal staphylococcal DHFR or with DHFRs from Gram-negative organisms is known. We recently embarked on a study of the mechanisms of Tp-resistance in multi-resistant staphylococci from various countries, and we are reporting the purification and N-terminal amino acid sequence of the Tp-sensitive DHFR from *Staphylococcus aureus*.

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2. EXPERIMENTAL

S. aureus ATCC 25923 is frequently used as a reference strain in susceptibility testing and was taken from our strain collection. *S. aureus* 853 and *S. aureus* 157/4696 are multiresistant clinical isolates, obtained from J. Braveny, Munich, and A.V. Graevenitz, Zürich. Strains were identified by their growth on mannitol-salt-agar, coagulase and DNase reaction and confirmed with the API-Staph system (API System SA).

SDS-PAGE gels were run using the Mini-Protean II equipment (BioRad) and the bands visualized using a silver stain kit (BioRad). Protein solutions were concentrated using the Centricon 10 system (Amicon). The DHFR assay was that of Baccanari and Joyner [7]. The assay mixture contained 0.1 mM NADPH, 0.06 mM dihydrofolate and 1 mM 2-mercaptoethanol in 50 mM imidazole-HCl buffer, pH 7.0. After addition of the enzyme, the fall in Δ_{340} was recorded using a Kontron Uvikon 860 spectrophotometer. A unit of DHFR activity was taken as the conversion of 1 μmol of dihydrofolate per minute.

Amino acid sequence determination was performed on an Applied Biosystems 470 A gas-phase sequenator. The phenylhydantoin amino acids were identified as described by Lottspeich [8].

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Minimum inhibitory concentrations (MIC) for antibiotics were determined by agar dilution on Mueller Hinton Agar [9].

3. RESULTS

3.1. *DHFR-purification*

S. aureus cells were grown in a 50 l fermenter in L-broth. For *S. aureus* 853 10 μg/ml Tp was added. Cells were broken by sonication after pretreatment with lysostaphin and lysozyme for 30 min at 30°C in a 0.05 M phosphate buffer, pH 7.0. After centrifugation at 10000 × *g* for 30 min, the supernatant was dialyzed overnight against 10 mM Tris buffer, pH 6.8, at 4°C and then loaded onto a column of Whatman DE-52 ion-exchange resin (20 × 1.7 cm). After washing with 5 column vols of buffer to remove non-bound proteins, the DHFR was eluted on a gradient of 0–0.25 M NaCl in the Tris buffer.

The DHFR activity of the resistant strains was eluted from the ion-exchange column in two peaks. In both cases the peak at 0.08 M NaCl (DHFR I) was approx. 100 times less susceptible to inhibition by Tp than the peak at 0.13 M NaCl (DHFR II). The reference strain ATCC 25923 displayed only one peak of DHFR activity (DHFR II, the Tp-sensitive, chromosomal DHFR) which eluted at 0.13 M NaCl. Fig.1 shows typical elution profiles from the DE-52 column. The specific activity of the DHFR is increased ten-fold by this step, but the yield is low. It is not clear whether this represents a loss of protein on the column or an inactivation of the enzyme.

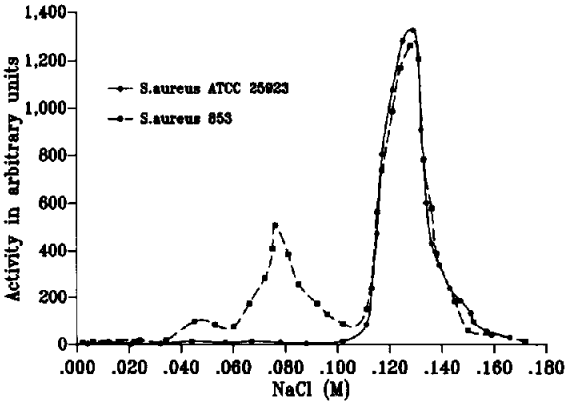

Fig.1. Elution of DHFR activity from a DE-52 column with salt gradient.

Table 1 compares the Tp-sensitivity of DHFR I and II and the antibiotic sensitivity of the three strains used in this study. Table 2 gives a more detailed comparison of the two DHFR types from strain 157/4696.

Attempts to purify further the DHFR on a methotrexate (MTX) affinity column failed as both enzymes I and II bound too firmly. However, a Blue Sepharose column proved suitable for the Tp-sensitive peak II, with a recovery of 70% and a further 40-fold increase in specific activity. The pooled fractions from the DE-52 column were diluted with an equal volume of the Tris buffer, in order to lower the salt concentration, and loaded onto a 8 × 1.6 cm column of Blue Sepharose (Pharmacia). The majority of the bound protein could be eluted by washing with 0.1 M KCl in the Tris buffer, and the DHFR was specifically eluted by adding 2 mM folate. In fig.2 a silver stained SDS-PAGE gel is shown. From this a greater than 95% purity of DHFR II is deduced. It was relatively unstable in its purified form and tended to precipitate irreversibly on dialysis to a lower salt concentration. This was prevented by introducing the non-ionic detergent Nonidet P-40 as the salt was dialyzed out to render the solution suitable for N-terminal amino acid analysis.

Table 1

Antibiotic susceptibility of three *S. aureus* strains and Tp sensitivity of DHFR I and II

Strain	MIC (μg/ml)[a]			IC_{50} (μM) for Tp	
	Tp	Meth	Pen	DHFR I	DHFR II
ATCC 25923	0.5	2	0.06	–	0.043
157/4696	>1024	4	128	6.7	0.044
853	512	64	128	7.0	0.062

[a] Meth, methicillin; Pen, penicillin G

Table 2

Comparison of DHFR I and II from *S. aureus* 157/4696

Property	DHFR I	DHFR II
IC_{50} (Tp)	6.7 μM	0.044 μM
IC_{50} (MTX)	2.25 nM	1.1 nM
K_m (DHF)	3.0 μM	4.0 μM
K_m (NADPH)	9.9 μM	6.3 μM
K_i (Tp)	0.88 μM	0.0094 μM
K_i (MTX)	0.12 nM	0.011 nM
Molecular mass	20 kDa	20 kDa

The Tp-resistant DHFR I could not be purified by the above method as it was not removed from the Blue Sepharose by salt concentrations up to 1 M or folate up to 10 mM. It could be removed by chaotropic agents, but not specifically. Further efforts are being made to purify this enzyme.

As can be seen from fig.2, the Tp-sensitive DHFR has a molecular mass of slightly under 20 kDa. Although the resistant enzyme has not yet been purified, the two activities were not separable by gel-filtration chromatography, suggesting that DHFR I is also of this molecular mass. The above purification method was applied to the Tp-sensitive enzymes from all three strains with equal success, and is summarized in table 3.

The purified, chromosomally coded DHFRs were subjected to automated N-terminal amino acid analysis. Several sequencing runs were carried out and the sequence, as shown in fig.3 was established for *S. aureus* 157/4696. Identical amino acid sequences were found for strain ATCC 25923 (sequenced up to residue 16) and strain 853 (sequenced up to residue 24).

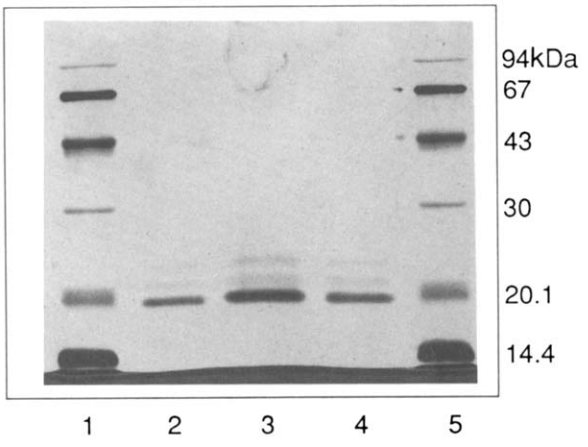

Fig.2. Silver stained SDS-PAGE gel of purified DHFR II from *S. aureus* 157/4696 eluted as described from the Blue Sepharose column (lanes 2,3,4). Lanes 1 and 5 are molecular mass standards.

Table 3

Purification of *S. aureus* DHFRs

Step	Total activity (units)	Specific activity (U/mg)	Yield (%)
Crude extract	72.4	0.050	–
DE-52	26.3	0.56	30.4[a]
Blue Sepharose	18.7	22.1	21.6[a]

[a] Yield based on total DHFR activity. The Tp-sensitive enzyme contributed between 60 and 80% of the total activity

4. DISCUSSION

An efficient two-step method enabled us to purify several Tp-sensitive, chromosomally encoded DHFRs to homogeneity. The highly hydrophobic nature of both staphylococcal enzymes is unusual among DHFRs and necessitated the use of a non-ionic detergent, Nonidet NP-40, to prevent irreversible precipitation. Despite this difficulty, up to 35 amino acid residues could be determined and all strains showed an identical sequence. A search for relationships to other known DHFRs showed best correlation with the Tp-sensitive, chromosomal *E. coli* DHFR [10]. 15 residues are identical, and considering conservative replacements according to Dayhoff et al. [11], there is a 69% sequence homology.

The corresponding Tp-resistant DHFR I exhibits several interesting properties. It is only moderately resistant to Tp, highly sensitive to MTX and has the same size as the Tp-sensitive enzyme. It differs, however, in its affinity for Blue Sepharose and DE-52. One could speculate therefore that this plasmid-encoded enzyme has evolved from the Tp-

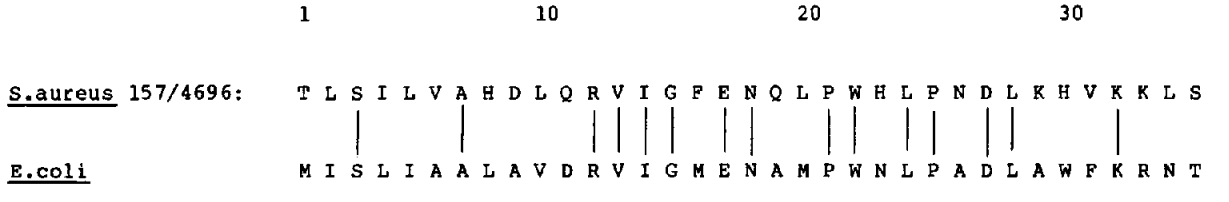

Fig.3. Comparison of the N-terminal amino acid sequence of *S. aureus* 157/4696 chromosomal DHFR and the *E. coli* DHFR taken from [10].

sensitive staphylococcal DHFR and is not a totally different species, as are the most important Tp-resistant plasmid-derived DHFRs from Gram-negatives [12]. Since the IC_{50} values for Tp for the pSK1 DHFR (50 μM [6]) contrast with 6.7 μM for the Tp-resistant *S. aureus* 157/4696, it remains an open question at present whether more than one type of plasmid-derived DHFRs prevail in *S. aureus*. pSK1 DHFR has been isolated from Australian strains, our strains were isolates from Central Europe. These questions can only be resolved by further studies. Unfortunately, the resistant DHFR could not yet be purified sufficiently well with the technique applied to the Tp-sensitive enzyme. Since the gene for the Tp-resistant DHFR was found to reside on plasmids (unpublished), efforts to clone this gene and efficiently express it for better purification and characterization are now under way.

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