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STREPTOMYCIN BIOSYNTHESIS

I. EXISTENCE OF TWO AMIDINOTRANSFERASE ACTIVITIES IN *STREPTOMYCES GRISEUS**

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SUMMARY

1. A cell-free extract with amidinotransferase activity (L-arginine:X amidinotransferase, EC class 2.1.4.) was obtained from frozen mycelia of *Streptomyces griseus* by shaking at 25° with 3 vol. of 0.1 M phosphate buffer (pH 7.4) containing 3 mg/ml EDTA.

2. By thermal inactivation of the cell-free extract at 45° two amidinotransferase activities were detected, distinguishable by their temperature sensitivities.

3. The enzymes were purified 4-fold by DEAE-Sephadex adsorption, $(NH_4)_2SO_4$ fractionation and column chromatography on the same Sephadex.

4. In the column chromatography two protein peaks were obtained, both with amidinotransferase activities, which correspond to the ones detected by thermal inactivation.

5. The question is discussed whether these two amidinotransferase activities correspond to two isoenzymes or to active subunits of a single enzyme, or whether they are two different enzymes with their specific physiological substrates, *O*-phosphoryl-*scyllo*-inosamine and *O*-phosphoryl-*N*-amidinostreptamine, both precursors of the streptomycin molecule.

INTRODUCTION

The enzyme L-arginine:X amidinotransferase (EC class 2.1.4.), discovered by WALKER[1,2] in streptomycin-producing strains of Streptomyces, catalyzes the transference of the amidine group from the donor L-arginine to the acceptor X. This acceptor X can be *O*-phosphoryl-*scyllo*-inosamine or *O*-phosphoryl-*N*-amidinostreptamine and the corresponding transamidination products *O*-phosphoryl-*N*-amidino-

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scyllo-inosamine or *O*-phosphorylstreptidine, respectively, precursors of the streptomycin molecule[3,4].

The present paper reports a simple method of extracting this enzyme from *Streptomyces griseus* mycelia and the demonstration of the existence of two amidinotransferase activities in it.

MATERIALS AND METHODS

Assay for amidinotransferase activity

Amidinotransferase activity was measured by the method of WALKER[5], by incubating the enzyme with L-arginine and hydroxylamine, as formamidine group donor and acceptor, respectively, and determining the hydroxyguanidine formed in the transamidination reaction with the complex $Na_3(Fe(CN)_5NH_3)$. A 0.1% solution of $Na_3(Fe(CN)_5NH_3)$ was used instead of the 1% solution employed by WALKER[5], because the colour stability was much greater with the more dilute solution. The readings were made 70 min after the addition of the reagent. The complex $Na_3(Fe(CN)_5NH_3)$ was prepared according to the method of HOFMAN[6] and the working standard hydroxyguanidine sulphate as described by WALKER AND WALKER[7]. One unit of enzyme activity is defined as the amount of enzyme which catalyzes the formation of 1 μmole of hydroxyguanidine in 1 min at 37°, under the assay conditions. The specific activity is expressed in units/mg of protein. Protein was determined by the method of LOWRY *et al.*[8].

Enzyme extract

Mycelia of about 100 h growth of streptomycin-producing *Streptomyces griseus* A-14 (Antibioticos, S.A. collection) grown on a soybean meal and glucose medium in an industrial fermentor were harvested by centrifugation and washed several times with distilled water, selecting the clean mycelia after each washing. Finally the wet mycelia were frozen at −20°. After 4 days, the frozen mycelia were left to thaw at room temperature. 3 ml of 0.1 M potassium phosphate buffer, pH 7.4, containing 3 mg/ml EDTA were added per g of wet mycelia and the suspension shaken in a rotary shaker at 25° for 3 h. Then it was diluted with about an equal volume of the same buffer and left overnight at 1°. The diluted suspension was centrifuged at 1° and 23 000 × *g* for 1 h. The lysed mucous mycelia were discarded.

Thermal inactivation of the enzyme extract

Aliquots of the enzyme extract were kept at a constant temperature of 45° and 60° for 1 h; 0.5-ml samples were taken every 10 min and their amidinotransferase activity was measured immediately.

First DEAE-Sephadex adsorption

DEAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden) was prepared as described by BERGMEYER *et al.*[9]. All the following operations were carried out at 1°. 1 l of DEAE-Sephadex was added with gentle stirring to 1 l of enzyme extract and the stirring continued for 30 min. Then the Sephadex was collected in a Buchner funnel by suction and the liquid discarded. The adsorption of the amidinotransferase activity is practically quantitative. The adsorbed enzyme was eluted with 500 ml of

0.1 M phosphate buffer, pH 7.6, containing 2 M NaCl by stirring for 30 min. The resin, collected in a Buchner funnel, was washed with a little eluting solution and discarded.

First $(NH_4)_2SO_4$ fractionation

Solid $(NH_4)_2SO_4$ was added to the 640 ml of eluate to give a final concentration of 2 M and stirred for 1 h. The practically inactive precipitate was centrifuged off at 23 000 × *g* for 30 min and discarded. The supernatant fluid was brought to 3 M by further addition of solid ammonium sulphate and stirred for 1 h. The active precipitate was centrifuged off at 23 000 × *g* for 1 h, dissolved in about 70 ml of distilled water and any insoluble material removed by centrifugation at 23 000 × *g* for 15 min. The supernatant fluid was dialysed for 2 h against running distilled water (about 25 l).

Second DEAE-Sephadex adsorption

The dialysed enzyme solution was diluted to 100 ml with distilled water and then the operation was repeated as in the first DEAE-Sephadex adsorption, but with only $^1/_{10}$ of the quantities indicated.

Second $(NH_4)_2SO_4$ fractionation

Solid $(NH_4)_2SO_4$ was added to the 100 ml of eluate up to a final concentration of 2 M, and stirred for 1 h. The precipitate was centrifuged off at 23 000 × *g* for 30 min and discarded. The supernatant fluid was made 3 M by further addition of $(NH_4)_2SO_4$ and stirred for 1 h. The precipitate was centrifuged off at 23 000 × *g* for 1 h and dissolved in about 30 ml of distilled water.

Any insoluble material was removed by centrifugation at 23 000 × *g* for 15 min. The enzyme solution was dialysed against running distilled water for 2 h (about 25 l).

Chromatography on DEAE-Sephadex

DEAE-Sephadex A-50 equilibrated with 0.05 M phosphate buffer, pH 7.6, was used for the filling of the chromatographic column (2 cm × 20 cm). The diffusate was adsorbed on to the column. During the adsorption phase itself, the formation of two visible zones of light yellow colour was observed. One of them remained strongly adsorbed at the top of the column and the other migrated with the front of the solvent.

At the end of the adsorption this zone was situated approximately half-way down the column and was eluted with 0.05 M phosphate buffer, pH 7.6. When it was completely eluted, then the strongly adsorbed zone was eluted with the same buffer containing 1 M NaCl. Both principal zones were active. 5-ml fractions were collected during the chromatography, activity and protein being determined in each fraction.

In previous experiments a larger column relative to the amount of protein had been employed with a ratio g protein/ml Sephadex smaller than 1/200 and the formation of the two zones was observed. Afterwards, approximately a double ratio protein/adsorbent was used in order to take advantage of the capacity of the column.

The first eluted active zone was designated as amidinotransferase I and the second one as amidinotransferase II.

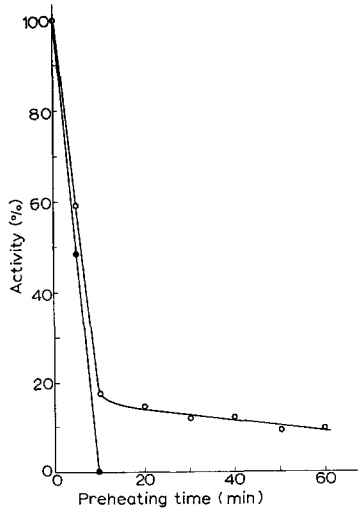
Thermal inactivation of the amidinotransferases I and II

6 ml of solution of the first zone, eluted from the chromatographic column (amidinotransferase I) with 0.05 M phosphate buffer, pH 7.6, were made up to 1 M NaCl by adding solid NaCl. This was done in order to have the solution of amidinotransferase I in the same ionic conditions as the second zone solution (amidinotransferase II), which was eluted with a 1 M NaCl in the mentioned buffer. The protein concentration was 7.0 mg/ml. This solution was kept at a constant temperature of 45° for 1 h. 0.5 ml samples were taken every 10 min, and their amidinotransferase activity measured immediately.

6 ml of solution of the second zone, eluted from the chromatographic column (amidinotransferase II) with 1 M NaCl in 0.05 M phosphate buffer, pH 7.6, and protein concentration of 7.6 mg/ml were submitted to the same experiment.

Calculation of K_m for the amidinotransferases

The kinetic constant K_m for the amidinotransferases I and II was calculated from Lineweaver–Burk plots. As enzyme solutions of amidinotransferase I and II, the first and second zones eluted from the chromatographic column, respectively,



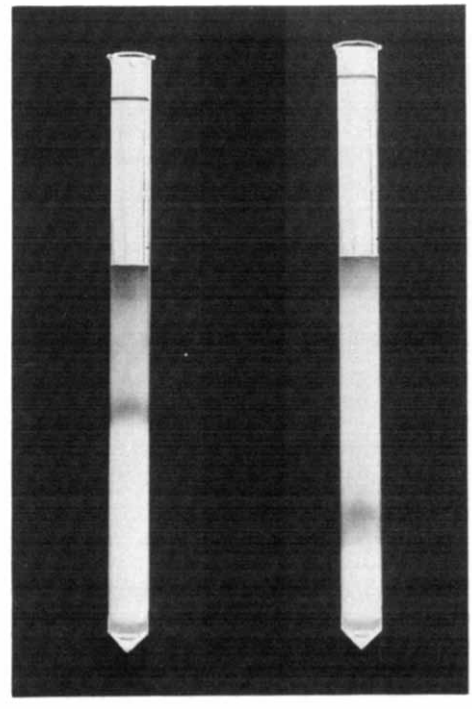


Fig. 1. Thermal inactivation of the enzyme extract. ○—○, preheating at 45°; ●—●, at 60°. The initial activity of the extract was $44 \cdot 10^{-3}$ units/ml and the protein concentration7.1 mg/ml.

Fig. 2. Chromatographic separation of two amidinotransferases on a DEAE-Sephadex column (2 cm × 20 cm) charged with 330 mg of protein. Left: final of the adsorption phase; first zone in the middle of the column (amidinotransferase I) and second zone strongly adsorbed at the top of the column (amidinotransferase II). Right: elution phase; first zone near the bottom of the column.

were used. The activities of the two enzymes were determined in the concentration range 0.1–0.01 M of the substrate L-arginine. The straight lines which best fitted the experimental data were calculated by the method of least squares.

RESULTS

Thermal inactivation of the enzyme extract

Thermal inactivation experiments at 45° and 60° on the enzyme extract, illustrated in Fig. 1, provided the first indication of two amidinotransferase activities, distinguishable by their temperature sensitivity. The more heat-labile enzyme is

TABLE I

SUMMARY OF A TYPICAL PURIFICATION AND SEPARATION OF TWO AMIDINOTRANSFERASES FROM *Streptomyces griseus* MYCELIA

Only the fractions of greatest specific activity were taken into account at the chromatography stage.

Stage of purification	*Total vol. (ml)*	*Total units* $\times 10^3$	*Total protein (mg)*	*Specific activity* $\times 10^3$ *(units/mg protein)*	*Enrich-ment (-fold)*	*Total recovery (%)*
Enzyme extract	1000	37000	7200	5.1	—	100
1st DEAE–Sephadex adsorption	640	34560	4416	7.8	1.5	93
1st $(NH_4)_2SO_4$ fractionation	75	17700	1268	13.9	2.7	48
2nd DEAE–Sephadex adsorption	100	14000	980	14.2	2.8	38
2nd $(NH_4)_2SO_4$ fractionation	34	10200	640	15.9	3.1	27
Chromatography on DEAE–Sephadex						
Amidinotransferase I (1st zone)	5	170	8	21.2	4.1	0.5
Amidinotransferase II (2nd zone)	5	1800	90	20.0	3.9	5

practically inactivated after 15 min at 45°, but the less heat-labile enzyme is not inactivated after 1 h at 45°. Both enzymes are completely inactivated after 10 min at 60°. This is a similar case to the two ribose-5-*P* isomerase activities described by DAVID AND WIESMEYER[10].

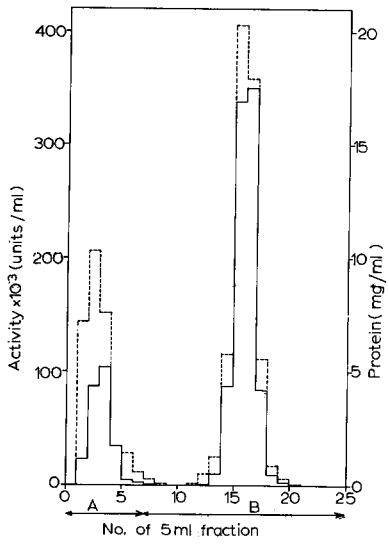
A photograph of the two active chromatographic zones is shown in Fig. 2.

The results of a typical purification and separation of the two amidinotransferases are illustrated in Table I. The two amidinotransferases were designated amidinotransferase I (first eluted zone) and amidinotransferase II (second eluted zone).

Fig. 3 shows a pattern of the column chromatography on DEAE-Sephadex of the two amidinotransferases.

Thermal inactivation of the amidinotransferases I and II

Fig. 4 shows the thermal inactivation of the two separated amidinotransferases



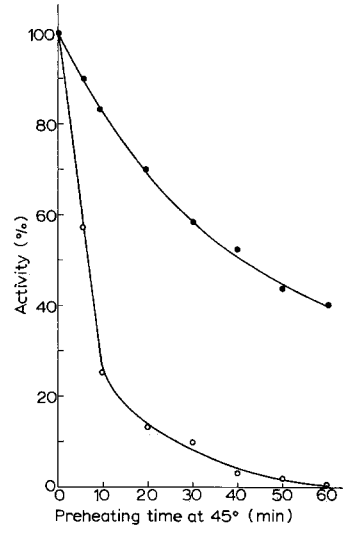


Fig. 3. Chromatographic separation of two amidinotransferases of *Streptomyces griseus* on a DEAE–Sephadex column. , amidinotransferase activity (units × 10^3/ml); – – – – –, protein (mg/ml). Buffers: A, 0.05 M phosphate buffer (pH 7.6); B, 0.05 M phosphate buffer (pH 7.6) containing 1 M NaCl.

Fig. 4. Thermal inactivation of the amidinotransferases I and II at 45°. ○—○, amidinotransferase I; ●—●, amidinotransferase II. Details described in MATERIALS AND METHODS.

kept at a constant temperature of 45° for 1 h. It can be seen that while amidinotransferase I was completely inactivated after 1 h, amidinotransferase II retained about 40% of its initial activity after that time.

Calculation of K_m for the amidinotransferases

From the Lineweaver–Burk plots, illustrated in Fig. 5, a K_m of 37.7 mM for amidinotransferase I and a K_m of 8.4 mM for amidinotransferase II were calculated.

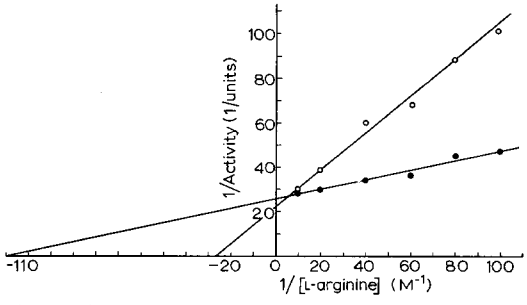


Fig. 5. Lineweaver–Burk plots for the calculation of the K_m for the amidinotransferases. ○—○, amidinotransferase I; ●—●, amidinotransferase II. Details described in MATERIALS AND METHODS.

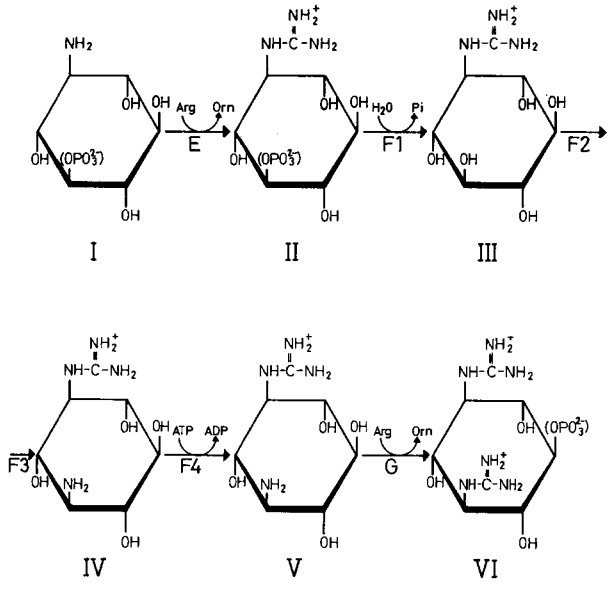


Fig. 6. Scheme proposed by WALKER AND WALKER[11] for the biosynthesis of *O*-phosphorylstreptidine from *O*-phosphoryl-*scyllo*-inosamine. I, *O*-phosphoryl-*scyllo*-inosamine; II, *O*-phosphoryl-*N*-amidino-*scyllo*-inosamine; III, *N*-amidino-*scyllo*-inosamine; IV, *N*-amidinostreptamine; V, *O*-phosphoryl-*N*-amidinostreptamine; VI, *O*-phosphorylstreptidine.

DISCUSSION

WALKER AND WALKER[11] have proposed the following scheme of Fig. 6 for the biosynthesis of *O*-phosphorylstreptidine from *O*-phosphoryl-*scyllo*-inosamine. This biosynthetic scheme implies two transamidination reactions E and G. WALKER AND WALKER[4] assume "as a working hypothesis, until contrary evidence is obtained, that Steps E and G are catalyzed by a single enzyme, L-arginine:*O*-phosphoryl-*N*-amidinostreptamine amidinotransferase (EC class 2.1.4.)".

Now the question arises as to whether the two amidinotransferase activities from *Streptomyces griseus*, whose discovery is reported in this paper, correspond to two isozymes or to active subunits of a single enzyme or whether they are two different enzymes with their specific physiological substrates *O*-phosphoryl-*scyllo*-inosamine and *O*-phosphoryl-*N*-amidinostreptamine.

All these points are under investigation.

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