IDENTIFICATION OF AMINO CITRIC ACID IN BIOLOGICAL PEPTIDES

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1. Introduction

A group of relatively small ribonucleoproteins which is widely abundant in nature was described [17] to act as highly specific antigen [2–5] as well as influencing DNA transcription depending on the species and the organ from which they are prepared [3].

The protein moiety of these molecules is characterized by higher amounts of aspartic acid and glutamic acid compared to lysine and arginine. A surprising feature of the amino acid analysis was the existence of a substance that eluted prior to aspartic acid and could not be assigned to one of the known amino acids that occur in nature. It will be shown that the unknown substance was identified as amino citric acid.

2. Materials and methods

Calf thymus ribonucleoprotein was isolated as in [1]. (1s,2s) amino citric acid [6] was kindly supplied by Dr Gutmann of Hofmann-La Roche, Basel.

2.1. *Acidic hydrolysis*

Dry ribonucleoprotein (250 mg) was dissolved in 1 ml distilled water and hydrolyzed in 10 M HCl at 54°C for 48 h.

2.2. *High voltage electrophoresis*

Electrophoresis was carried out on Whatman M3 preparative paper for 2–3 h at 15–20 V/cm using 0.1 M acetic acid–pyridine buffer (pH 3.6) with 4% phenol additive. Synthetic amino citric acid, 10 μl (10 μg)/cm, was applied as a marker at the margins of the paper. On staining the margins with ninhydrin (1s,2s) amino citric acid yielded a yellow coloured band. The corresponding zone of the paper was cut out, eluted with distilled water and lyophilized. All following experiments were conducted on electrophoretically isolated material.

2.3. *Thin-layer chromatography after periodic acid oxidation*

Periodic acid oxidation of hydroxyaminoacids results in formation of ammonia, glyoxylic acid, and characteristic aldehydes and keto acids [7].

The lyophilizate obtained from 250 mg ribonucleoprotein was dissolved in 20 μl H_2O and 10 μl of a 1% periodic acid solution added. After shaking the solution for 2 min at room temperature it was loaded on Merck DC 60 plastic foils and developed using ethanol water (33:67). A solution of 10 μg (1s,2s) amino citric acid in 20 μl H_2O and 10 μl 1% periodic acid was treated in the same way. After separation the foils were dried and sprayed with 2,4-dinitrophenylhydrazine. After heating in an incubator for 5 min at 80°C the substances were vizualized by staining with 5% potassium hydroxide in ethanol. Ammonia was detected as a reaction product of periodic acid oxidation by reaction with Nessler's reagent.

2.4. *Mass spectrometry*

A Laser-Micro-Mass-Analyzer (LAMMA) [8] was used for the identification of the amino citric acid. Samples were prepared by dripping an aqueous solution onto a formvar-coated electron microscopical grid followed by vacuum drying. 1 μl of a 0.1 mg/ml solution was sufficient for the recording of some hundred mass spectra.

3. Results

Fig.1 shows the amino acid profile of a peptide from calf thymus RNP after acid hydrolysis. The

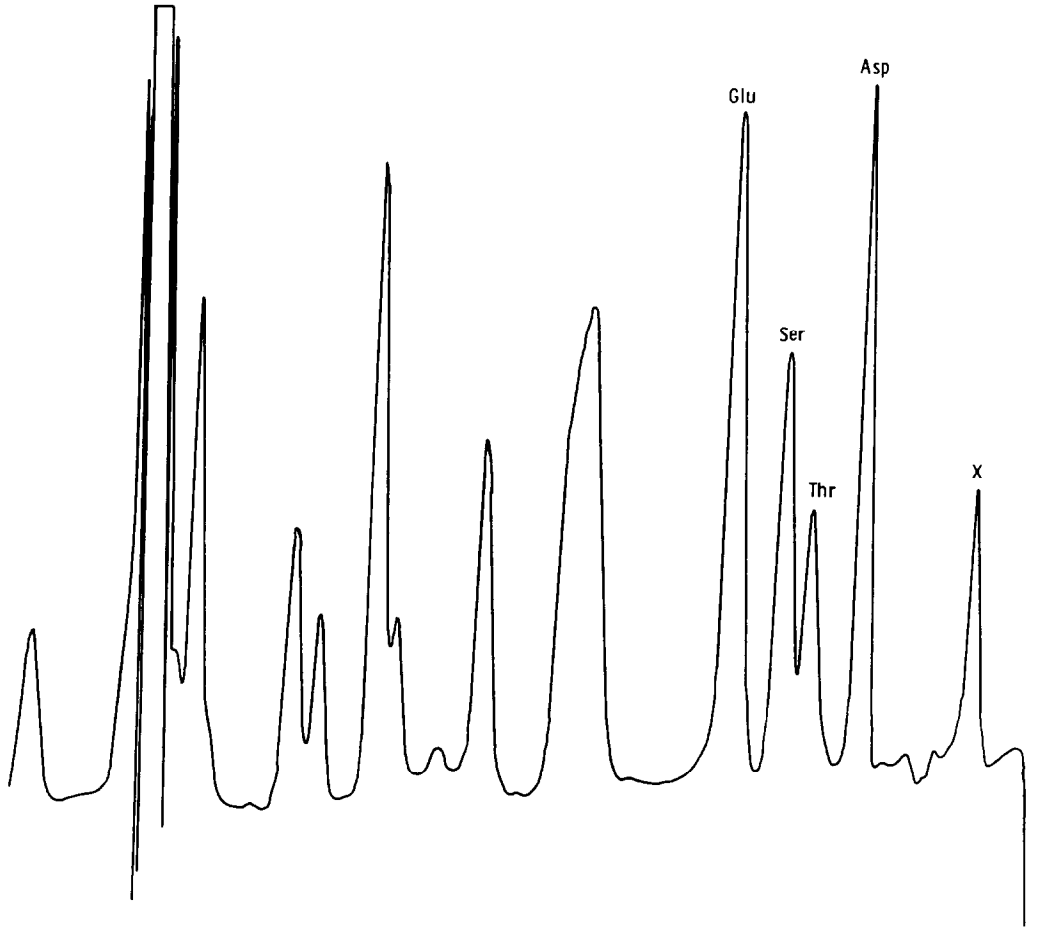

Fig.1. Amino acid analysis of calf thymus RNP total hydrolasate. Hydrolysis was in 6 M HCl at 110°C for 24 h. Separation was carried out with a Biocal amino acid analyzer. Asp, aspartic acid; Thr, threonine; Ser, serine; Glu, glutamic acid; X, unknown substance.

peak labelled 'X' of the unknown substance can be clearly identified. Its elution time was 10.4 min, that of aspartic acid 20.5 min. The peak 'X' eluted also prior to γ-carboxyglutamic acid, hydroxyaspartic acid, and cysteic acid and could thus clearly be differentiated from these amino acids (not shown).

Fig.2. illustrates the high-voltage electrophoretic pattern of a hydrolysate of calf thymus RNP. A slightly stained band is seen showing the same electrophoretic mobility as spots of authentical (1s,2s) amino citric acid at the margins. This result remained unaffected by pH change from 3.6–5.7 or 1.9. High-voltage electrophoresis was then used for the purification and further identification of this material. The maximum yields of 5–25 μg were obtained by direct hydrolysis of 250 mg ribonucleoprotein without pretreatment.

As shown in fig.3 the synthetic and the unknown substance moved also identically (and produced the characteristic yellow colour after ninhydrin staining) on thin-layer chromatography. 31 samples of the unknown substances isolated by high-voltage electrophoresis confirmed this result.

The identity of the unknown substance with synthetic amino citric acid was further supported by analysis of the reaction products after periodic acid oxidation. As shown in fig.4 this type of oxidation of both substances yielded mainly a keto acid that runs identically to oxaloacetic acid on silica gel. The smaller band appearing below the band of oxaloacetic acid (fig.4, lane 4) was identified as glyoxylic acid. Ammonia, another expected product of amino citric acid degradation was detected in separate experiments

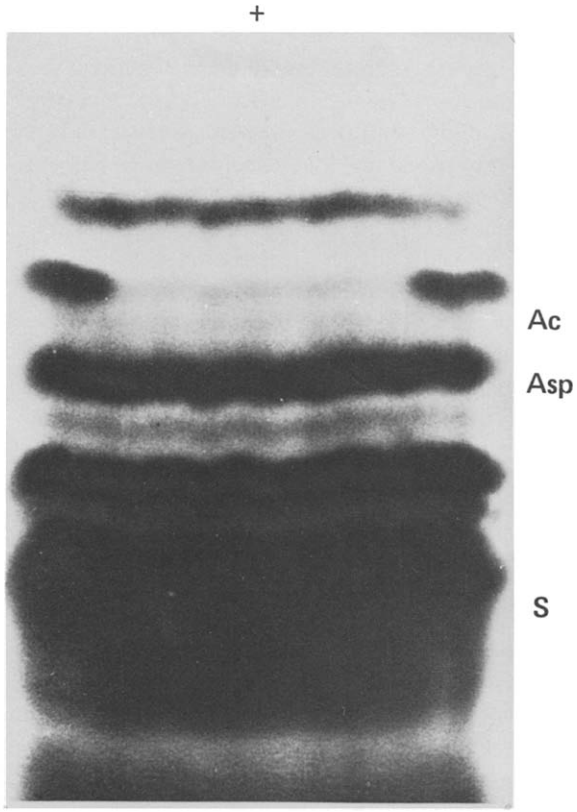

Fig.2. High-voltage paper electrophoresis of acid hydrolysate of calf thymus ribonucleoprotein. 250 mg calf thymus RNP were hydrolysed and subjected to electrophoresis as in section 2. AC, amino citric acid; Asp, aspartic acid; S, start.

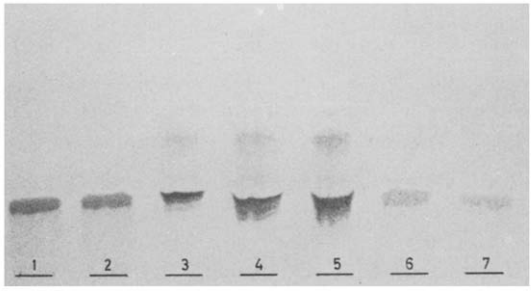

Fig.3. Migration of synthetic (1s,2s) amino citric acid and of unknown compound on thin-layer chromatography. The material from RNP hydrolysates purified by electrophoresis was chromatographed on silica gel DC 60 foils (Merck) using ethanol water (33:67) as liquid phase. Ninhydrin stain. (1,2) Synthetic (1s,2s) amino citric acid (20 μg); (3–5) different eluted bands after different electrophoretic runs; (6,7) 1:2 dilution of bands (3,4) with H_2O. RF amino citric acid 0.24.

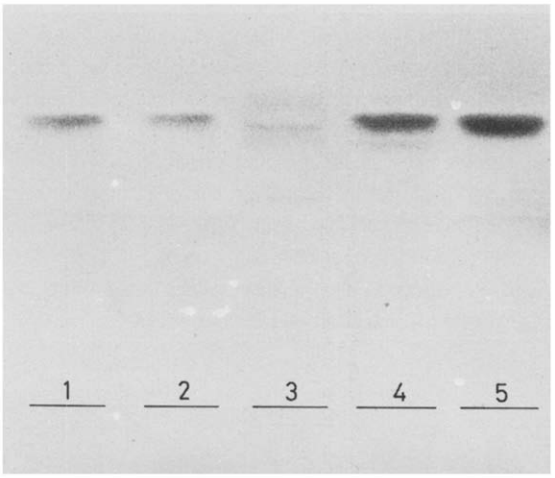

Fig.4. Thin-layer chromatography of periodate oxidation products of synthetic amino citric acid and unknown material compound. For experimental details see section 2. (1,5) Oxaloacetic acid (5 μg); (2) synthetic amino citric acid (20 μg); (3,4) different samples of purified natural compound.

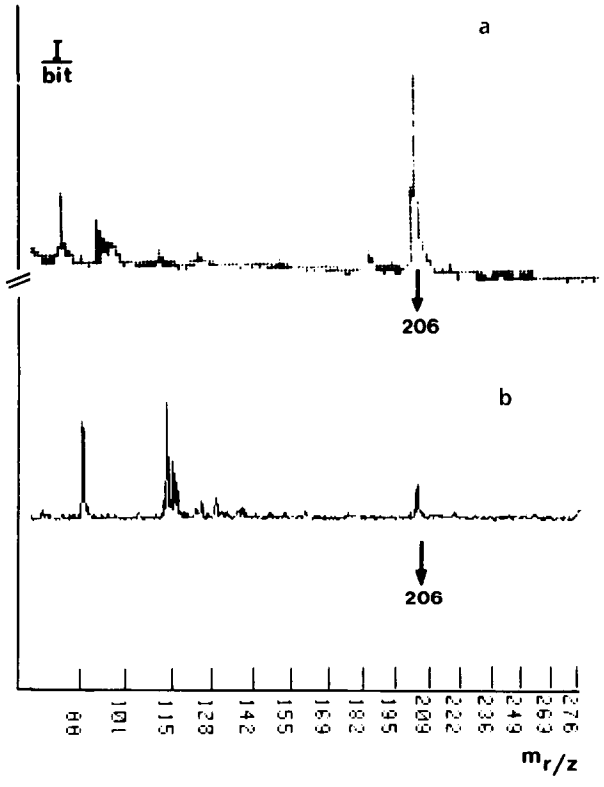

Fig.5. Negative ion spectra of <1pg synthetic amino citric acid (a) and electrophoretically purified unknown substance (b).

in samples incubated with periodate.

Clear proof for the identity of the atypical new amino acid from RNP with amino citric acid was finally obtained by mass spectrometry. The molecule can be identified by its deprotonated ion at $m_{r/Z} = 206$ in the negative spectra. An acidic hydrolysate of calf thymus ribonucleoprotein displayed a distinct though small peak at this mass number (not shown). This peak was markedly increased in the spectrogram (fig.5b) of a purified sample of the substance. The mass spectrum of synthetic amino citric acid is shown in fig.5a.

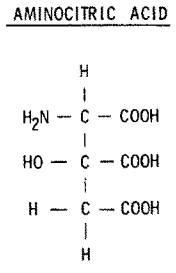

Fig.6.

In positive ion spectra synthetic (1s,2s) amino citric acid was recognized after addition of Ca^{2+} by a peak at mass number $m_{r/Z} = 247$ while this treatment strongly decreased the abundance of the negative ion of the free amino acid. After oxalic acid precipitation of the Ca^{2+} salt the $m_{r/Z} = 247^{+}$ peak vanished while the $m_{r/Z} = 206^{-}$ was recovered. The same was found to hold for the unknown substance.

4. Discussion

This report describes the isolation of a hitherto unknown amino acid from calf thymus ribonucleoprotein and its identification as amino citric acid (fig.6). From 100 mg ribonucleoprotein, 1–5 μg purified amino citric acid was obtained. Owing to the small amounts the stereochemical configuration of the compound could not be determined. We have observed that ribonucleoproteins from bovine and human spleen, *E. coli*, and *Salmonella typhi* bacteria also contain amino citric acid though in smaller amounts than calf thymus. The physiological significance of this new amino acid is not clear at present yet its occurence even in bacterial ribonucleoprotein points to some biologically fundamental role of this constituent.

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