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Molecular weight analysis of isopenicillin N synthase by electrospray mass spectrometry

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The use of electrospray mass spectrometry as a tool in analytical biochemistry was illustrated by determination of the molecular weights of wildtype and recombinant isopenicillin N synthase (IPNS). The molecular weight of recombinant IPNS produced using an expression system which generated soluble protein was found to be between 38364 and 38376 Da, ca 60 mass units higher than that of the wildtype material, consistent with the presence of an additional N-terminal glycine in the former. Observed molecular weights were all ca 70 Da higher than that calculated from sequence information, consistent with the complexion of a partially hydrated iron atom to the enzyme during analysis.

Cephalosporium acremonium; Electrospray mass spectrometry; Isopenicillin N synthase; Molecular weight; Penicillin biosynthesis

1. INTRODUCTION

Isopenicillin N synthase (IPNS) is the pivotal enzyme in the biosynthesis of penicillins in that it catalyses the oxidative cyclisation of L-α-aminoadipoyl-L-cysteinyl-D-valine (*LLD*-ACV) to isopenicillin N (IPN). The enzyme is a ferrous dependent oxygenase and requires L-ascorbate and dithiothreitol (DTT) for optimal activity. In the course of the past 2 decades both ourselves and others have been engaged in the isolation of the protein and cloning of the gene encoding this interesting enzyme [1]. The enzyme has been purified to homogeneity from *Cephalosporium acremonium* CO 728 and an N-terminal sequence determined [2]. Using this information, the gene for IPNS has been cloned and the enzyme was initially expressed in *Escherichia coli* JM109 transformed with pIT337, under control of the *trp* promoter [3]. N-terminal sequence analysis of this recombinant protein, which was expressed in soluble form, indicated that it contained an extra glycine residue at the N-terminus compared with the wild type material [4]. Using alternative expression systems employing the *trp* and *trc* promoters constitutive and inducible high level expression systems for the production of soluble IPNS have been developed [5].

The mass analysis of large biomolecules by electrospray ionisation into an atmospheric pressure ion source of a quadrupole mass spectrometer provides a rapid and sensitive method for the molecular weight determination of proteins and oligonucleotides [6-8] via the formation of a series of multiply charged ions from which the molecular weights are deduced by simple algorithms [6].

In this paper we illustrate the analytical power of electrospray mass spectrometry in biological chemistry, by determining the molecular weights of recombinant IPNS produced in *E. coli* strain NM544 using expression vectors under the control of the leftward promoter of phage λ *E. coli* NM544 / pIT353 [9] and *trp* promoter *E. coli* [5,10] and of the wildtype enzyme.

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2. EXPERIMENTAL

2.1. *Enzyme preparation and purification*

Recombinant IPNS was prepared in a soluble form using *E. coli* strain NM544 essentially according to the published procedures [3,5]. Wildtype IPNS was prepared and purified from *C. acremonium* CO728 according to a modified version of the published procedure [11]. *E. coli* strain NM544 transformed with pIT353 was lysed with lysozyme, DNA digested with DNase. Insoluble protein from inclusion bodies was solubilised with 7 M urea, the resultant solution was then dialysed against 50 mM Tris-HCl buffer pH 7.7 and thereafter purified essentially according to procedure [3]. Full details of the preparation of the recombinant IPNS used in this study will be published elsewhere.

Prior to analysis the enzymes were desalted and buffer exchanged to 20 mM Tris-HCl pH 8.0 by gel filtration, using a PD-10 (Pharmacia) column. The concentration of enzymes were adjusted to 10 mg/ml (260 pmol/μl) by ultrafiltration using a PM10 (Amicon) membrane.

2.2. *Molecular weight analysis*

The enzymes in 20 mM Tris-HCl were mixed with methanol and acetic acid, which was used as the proton source, to give a final concentration of 130 pmol/μl enzyme, 50% methanol, and 1% acetic acid. The sample solution (10 or 20 μl) was injected into the electrospray system of a prototype VG BIO Q quadrupole mass spec-

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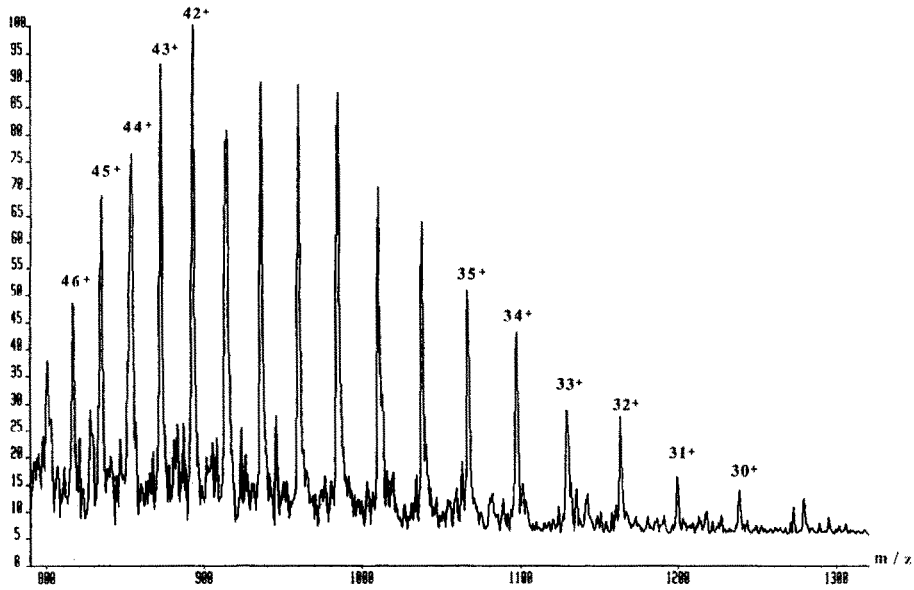

Fig. 1. Typical electrospray mass spectrum of highly purified IPNS strain A.

trometer via a loop injector at typical flow rate of 5 μl/min and scanned over a mass range of 700–1500 Da. The instrument was calibrated with myoglobin (M_w 16950.5).

3. RESULTS AND DISCUSSION

A typical electrospray mass spectrum of highly purified IPNS produced in a soluble form (strain A) [3,5] is shown in Fig. 1. A series of multiply charged ions $(M + Hn)^{n+}$ ranging from 30^+ to 47^+ was observed. The results from series of different IPNS preparations are shown in Table I.

Initially the molecular weight of IPNS strain A was examined using the electrospray technique. The results were reproducible over a number of experiments, utilising protein from four different preparations (Expts 1–4, Table I), indicating a molecular weight of between 38 364 and 38 376 Da, significantly higher than that calculated from the wildtype amino acid sequence plus glycine (vide infra) [4].

We then examined the spectrum of wildtype IPNS from *C. acremonium* CO 728 (Expts 5 and 6, Table I) and found the molecular weight was ca 60 mass unit less than the recombinant material (strain A), consistent with the absence of an N-terminal glycine residue in the wildtype material. Furthermore the mass spectrum of this enzyme was also ca 70 Da higher than that calculated from sequence data [3].

Table I

Molecular weight determination of IPNS's using electrospray mass spectrometry

Expt	Sample	n	Measured Av.M_w	σD	Calculated average mass		
					(i)	(ii)	(iii)
1	strain A-1	5	38364.2	1.3	*38301.1	38357.0	38375.0
2	strain A-2	5	38376.3	4.2			
3	strain A-3	3	38375.6	2.9			
4	strain A-4	4	38372.3	2.9			
5	wildtype-1	3	38309.1	4.0	**38244.2	38300.0	38318.0
6	wildtype-2	1	38314.8				
7(i)	strain B	1	38370.3		38301.1	38357.0	38375.0
7(ii)			38510.3		***38432.4	38488.2	38506.2

n = number of determinations; σD = standard deviation of each of the determinations; for calculated molecular weights (i) = M_w calculated from sequence information; (ii) = (i) + Fe (55.9); (iii) = (i) + Fe + H_2O (73.9). *N-terminal sequence was assumed to be: Gly-Ser-Val-Pro-Val-..., **N-terminal sequence was assumed to be: Ser-Val-Pro-Val-..., ***N-terminal sequence was assumed to be: Met-Gly-Ser-Val-Pro-Val-... and *not* to be N-formylated; M_w = 38 522 if fully formylated. Average mass calculated using the average of the isotopic masses weighted by abundance, i.e., C = 12.011, H = 1.008, N = 14.007, etc. This corresponds to the centroid of the molecular ion distribution.

The spectra of recombinant IPNS derived from *E. coli* NM544 / pIT353 (strain B) was more complex than those previously observed. Two series of multiply charged peaks were observed, one of which [Expt 7(i), Table I] was consistent with the result obtained for the IPNS strain A. The other series of peaks indicated a molecular weight approximately 140 Da higher which is consistent with the production of IPNS by strain B in which there was incomplete cleavage of an N-terminal methionine (possibly partially N-formylated, M_w of methionine: 131.2) to give two isozymes of IPNS. It is of interest that strain B produces IPNS in the form of cytoplasmic aggregates or inclusion bodies [12], which must be denatured in urea and refolded to give active IPNS, possibly explaining why there is incomplete cleavage of the N-terminal methionine in the latter case.

In each case, allowing for the N-terminal variations, the observed molecular weight was ca 70 mass units higher than that calculated from the known sequence information, IPNS has been shown to contain a single iron (II) atom [13, 14], thus 56 of the excess mass units can be accounted for by assuming the iron is bound to the protein during the mass spectrometric analysis. The calculated masses are still, however, 7–19 Da lower than the observed masses [calculation ii, Table I]. In view of the consistency of this mass difference over a range of preparations, we believe this difference can best be rationalized by the complexation of a water molecule to the iron atom in the enzyme. The variation between 7 and 19 Da in the measured mass may correspond to partial hydroxylation of the enzymes since the mass resolution on the polycharged ions is insufficient to separate the two species.

In the catalytic conversion of *LLD*-ACV to IPN the complexed water molecule might be displaced by the thiol of *LLD*-ACV to give an iron-sulphur linkage as we have previously proposed [14], a hypothesis which has recently been supported by spectroscopic analyses of IPNS complexes [15].

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