Structural studies on the reaction of isopenicillin N synthase with the substrate analogue δ -(L- α -aminoadipoyl)-L-cysteinyl-D- α -aminobutyrate

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Isopenicillin N synthase (IPNS) is a non-haem iron(II) oxidase which catalyses the biosynthesis of isopenicillin N from the tripeptide δ -(L- α -aminoadipoyl)-L-cysteinyl-D-valine (ACV). Herein we report crystallographic studies to investigate the reaction of IPNS with the truncated substrate analogue δ -(L- α -aminoadipoyl)-L-cysteinyl-D- α -aminobutyrate (ACAb). It has been reported previously that this analogue gives rise to three β -lactam products when incubated with IPNS: two methyl penams and a cepham. Crystal structures of the IPNS–Fe(II)–ACAb and

IPNS–Fe(II)–ACAb–NO complexes have now been solved and are reported herein. These structures and modelling studies based on them shed light on the diminished product selectivity shown by IPNS in its reaction with ACAB and further rationalize the presence of certain key residues at the IPNS active site.

Key words: β -lactam antibiotic, non-haem iron enzyme, oxidase, penicillin biosynthesis.

INTRODUCTION

Isopenicillin N synthase (IPNS) is a non-haem iron oxidase with an essential role in the biosynthesis of all penicillin and cephalosporin antibiotics [1]. It mediates conversion of the linear tripeptide δ -(L- α -aminoadipoyl)-L-cysteinyl-D-valine (ACV; structure **1** in Figure 1) to bicyclic isopenicillin N (IPN; **2**) in a single step, believed to occur via a monocyclic β -lactam/iron(IV)-oxo intermediate **3** (Figure 1a) [2,3]. IPN represents a branch point in the scheme of penicillin and cephalosporin biosynthesis: transacylation of its side chain gives rise to hydrophobic penicillins [4,5] whereas epimerization at the remote α -aminoadipoyl centre [6] followed by ring expansion opens the way to the cephalosporins [7].

An extensive array of incubation studies has been undertaken with IPNS and a range of substrate analogues [8]. The truncated analogue δ -(L- α -aminoadipoyl)-L-cysteinyl-D- α -aminobutyrate (ACAb; **4**) lacks one methyl group relative to the isopropyl side chain of D-valine in the natural substrate. Reaction of ACAB with IPNS gave rise to three products: two epimeric C2-methyl penams (the α -methyl and β -methyl compounds; **5** and **6** respectively) and the cepham **7** (Figure 1b) [9,10]. A 1:7:3 product ratio of α -methyl penam/ β -methyl penam/cepham has been reported [9,11]. The cepham **7** represented the first non-penam product to be isolated from an IPNS-catalysed reaction, and its formation is indicative of lowered product selectivity during closure of the second ring. It has been suggested that the penam/cepham ratio is dictated by the relative heights of the energy barriers to reaction of an iron(IV)-oxo intermediate with primary and secondary C–H bonds in the substrate [11]. Furthermore, it has been postulated that the observed product ratio reflects relative occupancies of three different γ -methyl rotamers of the aminobutyric acid residue in a monocyclic β -lactam species derived from ACAB during IPNS catalysis [11,12].

ACAb analogues incorporating each isomer of stereospecifically deuterated aminobutyric acid, labelled at the β -carbon, have also been incubated with IPNS; the same β -methyl penam product **8** was isolated from both isomers of the starting material [13]. This result has been explained by considering secondary radical intermediates that are able to rotate freely in the IPNS active site. In contrast, labelling studies have shown that IPN formation from ACV is highly stereoselective with respect to closure of the thiazolidine ring [14], presumably because rotation of the isopropyl side chain is restricted relative to the ethyl group of ACAB. Site-directed mutagenesis has been used to reduce the steric demand around the IPNS active site, and while the mutant forms of the enzyme did not convert ACV to cepham products, the ratio of products obtained from ACAB was altered significantly [12].

The β -methyl penam **9** and cepham **10** (Figure 1c) which bear the D- α -aminoadipoyl side chain have been isolated from fermentations of *Streptomyces* ACC 13285 [15]. It seems probable that these natural products are derived via *in vivo* assembly of ACAB by a tripeptide synthetase, followed by IPNS-mediated cyclization and subsequent side-chain epimerization [1,15].

The crystallization of IPNS with iron(II) and ACV [16] opened new avenues of investigation into this enzyme, including the possibility of studying how product selectivity is achieved in a process involving highly reactive non-haem ferryl species. The aim of the present study was to use protein crystallography to investigate the incubation results previously obtained with ACAB and IPNS in solution. Here we report structures for the anaerobic IPNS–Fe(II)–ACAb complex and the complex of enzyme, iron(II), ACAB and NO as a structural analogue of molecular oxygen. We also discuss the outcome of modelling studies using the structure of the IPNS–Fe(II)–ACAB–NO complex to investigate the dynamics of ACAB binding in the active site. The results allow conclusions to be drawn regarding the reaction of

Abbreviations used: ACV, δ -(L- α -aminoadipoyl)-L-cysteinyl-D-valine; ACAB, δ -(L- α -aminoadipoyl)-L-cysteinyl-D- α -aminobutyrate; IPN, isopenicillin N; IPNS, isopenicillin N synthase; Wat, water molecule.

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Figure 1 Reactions of IPNS with ACV and ACAB

Shown are the reactions of IPNS with (a) ACV and (b) ACAB. (c) Structures of the penam (9) and cepham (10) natural products isolated from fermentations of *Streptomyces* ACC 13285 [15] and proposed to derive biosynthetically from ACAB. L-AA, L- δ -(α -aminoadipoyl); D-AA, D- δ -(α -aminoadipoyl).

IPNS with the truncated substrate analogue and offer insight into the mechanism of the enzyme with its natural substrate.

MATERIALS AND METHODS

Synthesis of ACAB

D- α -Aminobutyric acid was converted to its benzhydryl ester using *p*-toluenesulphonic acid and diphenyldiazomethane [17]. The benzhydryl ester was then coupled to a suitably protected aminoadipoyl-cysteine dipeptide using 1-(3-dimethylamino-propyl)-3-ethylcarbodi-imide hydrochloride ('EDCI') and 1-hydroxybenzotriazole hydrate ('HOBt') [18]. Finally, trifluoroacetic acid-mediated deprotection [19] afforded the crude product, which was purified by reversed-phase HPLC [octadecylsilane (250 mm $\times$ 10 mm), 10 mM NH₄HCO₃ in water/methanol as eluant (running time, 0–6 min, 2.5%; 6–14 min, 25%; 14–20 min, 2.5% methanol, v/v), 4 ml/min, λ = 254 nm, 5 absorbance units full scale ('AUFS'); retention time 4.9 min] to give purified tripeptide in its thiol form for crystallization with IPNS.

Crystallization and NO experiments

Crystals of IPNS-Fe(II)-ACAB were grown under anaerobic conditions as reported previously [20] with one minor modification. The general procedure for crystallizing IPNS with substrate analogues allows for an approx. 50-fold excess of substrate (2 mg:3.75 mg of protein). With ACAB, half this amount (1 mg) of substrate was used for the crystallization experiments. Nevertheless, crystals grew as expected and appeared identical in shape and size to those obtained previously with other substrate analogues [3,21].

Crystals to be used for determination of the IPNS-Fe(II)-ACAB structure were removed from the anaerobic environment, exchanged into cryoprotectant solution [20] and flash-frozen

in liquid nitrogen. To prepare the NO complex, crystals were exposed to NO as described previously [16] before being removed from the glove box, exchanged into cryoprotectant and flash-frozen.

Data collection and processing

Data were collected at 100 K using either one of the following: Cu-K α radiation from a Rigaku rotating anode generator operating at 60 kV and 70 mA and a 150 mm MAR Research image plate detector, at the Department of Molecular Biophysics, University of Oxford, Oxford, U.K. for the IPNS-Fe(II)-ACAB complex, or synchrotron radiation and a 345 mm MAR Research image plate detector at beamline BM14 of the European Synchrotron Research Facility (ESRF), Grenoble, France, for the IPNS-Fe(II)-ACAB-NO complex.

Data were indexed and integrated using DENZO [22] or MOSFLM [23] and scaled using either SCALEPACK [22] or SCALA from the CCP4 suite of programs [24]. Initial structures were generated by rigid body refinement of the protein atoms from the IPNS-Fe(II)-ACV model [16] against the new data. Further refinement was carried out using PROLSQ [24], REFMAC [24] and SHELXL [25]. The final round of refinement for both structures was performed with REFMAC. Electron density maps were interpreted using the program O [26]. The active-site iron atom, water molecules and a sulphate anion (from the crystallization buffer) were added in the course of refinement. There was electron density clearly visible for substrate-derived species throughout refinement, but substrate atoms were included in refinement sequentially, and only when their position could be unambiguously assigned from the electron density. Iron-ligand bond lengths were unrestrained throughout refinement. Crystallographic co-ordinates and structure factors have been deposited in the Brookhaven Protein Data Bank (PDB) under accession numbers 1OC1 for the IPNS-Fe(II)-ACAB structure and 1OBN for the IPNS-Fe(II)-ACAB-NO complex.

Figure 2 The active-site region of the IPNS-Fe(II)-ACAb complexes

Left-hand panel: the anaerobic IPNS-Fe(II)-ACAb complex with a $mF_o - DF_c$ omit map contoured at 2σ ; two orientations of the aminobutyrate methyl group are shown (as straight and segmented bonds respectively) for the reasons detailed in the text. Right-hand panel: the IPNS-Fe(II)-ACAb-NO complex with a $mF_o - DF_c$ omit map contoured at 2σ .

Colour figures were prepared using the programs MOLSCRIPT [27], BOBSCRIPT [28] and Raster3D [29].

Modelling studies

The relative energies of different conformations were calculated using the program QUANTA (Accelrys; <http://www.accelrys.com/index.html>), based on the interaction of Van der Waals surfaces. Amino acid side chains and other groups that might impinge upon the aminobutyrate γ -carbon were identified from the IPNS-Fe(II)-ACAb-NO crystal structure as Ile-187, Leu-223, Leu-231, Ser-281, Pro-283, Phe-285, Wat-182 (where Wat is water molecule), Wat-251, the NO ligand and the iron atom. Thus hydrogen atoms and van der Waals surfaces were added to these residues and to the $C\beta$ and $C\gamma$ atoms of the substrate using QUANTA. The side chain of the aminobutyrate residue was then rotated about its $C\alpha - C\beta$ bond in 5° intervals using the modelling program, and at each 5° point the $C\beta - C\gamma$ bond was also rotated to achieve the lowest energy conformation for the $N - C\alpha - C\beta - C\gamma$ torsion angle. A graph of torsion angle against relative energy was then plotted.

RESULTS AND DISCUSSION

The structure of the anaerobic IPNS-Fe(II)-ACAb complex

The structure of the IPNS-Fe(II)-ACAb complex was solved to $2.2 \text{ \AA}$ resolution from a crystal of hexagonal morphology [20] (Figure 2, left-hand panel, Table 1). The overall structure of the protein corresponds very closely to that of the IPNS-Fe(II)-ACV structure (root mean square difference of $0.132 \text{ \AA}$ for the amino acid main-chain atoms) [16], and the active-site region of this new complex for the most part resembles that of the ACV complex closely. The '2-His-1-carboxylate' triad of amino acids from the protein are iron ligands [31], as is the cysteinyl thiolate of the substrate, located *trans* to His-270, and also the conserved water ligand opposite His-214 (Wat-149 in PDB structure 1OC1).

As observed for ACV, ACAb is in a position to bind to the protein via electrostatic interactions with its two carboxylate groups. Thus the carboxylate of the aminoadipic acid side chain is held by a salt bridge to Arg-187, and the aminobutyryl carboxylate can form hydrogen bonds with Tyr-189, Ser-281 and to Arg-279 via a water molecule.

The structure of the ACAb complex in the region of the aminobutyrate side chain is clearly different to the ACV structure. Most significantly, there appears to be an additional water molecule bound to iron opposite Asp-216 (Wat-199). The side chain of the aminobutyrate residue has undergone rotation about its $C\beta - C\gamma$ bond to make room for the extra water ligand in this site. Thus the aminobutyrate β -carbon occupies a position similar to that of the valinyl β -carbon in the IPNS-Fe(II)-ACV-NO complex [in which rotation of the isopropyl by approx. 30° has occurred relative to the IPNS-Fe(II)-ACV structure, bringing the β -hydrogen towards iron] [16]. The γ -carbon of the aminobutyrate residue appears to exhibit multiple occupancy. In one orientation, this carbon is in approximately the same position as the valinyl pro-*R* methyl group in the IPNS-Fe(II)-ACV-NO complex; in the other, it takes a position equivalent to that of the pro-*S* methyl group in the IPNS-Fe(II)-ACV-NO complex. The omit electron density map shown (Figure 2, left-hand panel) does not contain strong electron density corresponding to the second of these orientations at the contour level used (2σ). However when the stringency of the map is lowered further, convincing density for the second γ -methyl occupancy is clearly visible, albeit in a noisier map. Moreover, the difference electron density map generated when the γ -carbon atom alone is omitted from refinement shows strong positive density corresponding to both methyl positions. It is on the basis of these observations that we propose partial occupancy of the two sites by the γ -carbon in the IPNS-Fe(II)-ACAb complex.

There are several alternative positions that the aminobutyrate γ -carbon might theoretically occupy. For the ensuing discussion these are defined relative to the positions of the methyl groups in

Table 1 Data collection and statistics

Complex . . .	IPNS-Fe(II)-ACAb		IPNS-Fe(II)-ACAb-NO	
X-ray source	Rigaku rotating anode		ESRF, BM14	
Wavelength λ (Å)	1.5418		0.8260	
Space group	P3 ₁ 21		P2 ₁ 2 ₁ 2 ₁	
Unit cell (a, b, c; Å)	101.0, 101.0, 115.7		46.7, 71.1, 100.8	
Resolution shell (Å)	19.00–2.20	2.32–2.20	19.55–1.30	1.36–1.30
Measurements	124191	16871	244112	22973
Mean $I/\sigma I$	13.7	3.3	13.0	3.1
Unique reflections	34696	5079	73254	9493
Completeness (%)	99.1	99.1	88.1	88.1
R_{merge} (%)*	8.3	40.0	6.5	34.4
R_{cryst} (%)†	17.1		16.8	
R_{free} (%)‡	19.9		18.9	
Root mean square deviation§	0.03 (2.4)		0.02 (1.9)	
B factors (Å ²)¶	15.2, 18.1, 50.9, 37.2		6.5, 8.2, 8.8, 22.4	
Residues	328		328	
Water molecules	205		225	
PDB accession no.	1OC1		1OBN	

* $R_{\text{merge}} = \sum_j \sum_h |I_{h,j} - \langle I_h \rangle| / \sum_j \sum_h \langle I_h \rangle \times 100$.

† $R_{\text{cryst}} = \sum ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum |F_{\text{obs}}| \times 100$.

‡ R_{free} , based on 4% of the total reflections.

§ Root mean square deviation from ideality for bonds (followed by the value for angles).

¶ Average B factors in order: main chain, side chain, substrate and solvent.

Figure 3 Possible positions for the methyl group of the aminobutyryl side chain, defined relative to the methyl positions in the IPNS-Fe(II)-ACV-NO structure

Conformation I ('methyl up') and conformation II ('methyl right') are the positions occupied by the two ACV methyl groups; conformation III ('methyl left') is the position filled by the valinyl β -hydrogen atom in the IPNS-Fe(II)-ACV-NO complex. The alternative naming system shown in parentheses ('methyl up/right/left') is that used previously elsewhere [11,12].

the IPNS-Fe(II)-ACV-NO complex (Figure 3) and referred to as conformation I, in which the aminobutyrate γ -carbon occupies the position of the valinyl pro-*S* methyl group, conformation II, in which it takes the place of the pro-*R* methyl carbon, and conformation III, in which it is oriented in the same direction as the isopropyl hydrogen atom in the IPNS-Fe(II)-ACV-NO complex. In the IPNS-Fe(II)-ACAb complex, the aminobutyrate γ -carbon appears to sit partly in conformation I and partly in conformation II.

The structure of the IPNS-Fe(II)-ACAb complex indicates that without the second methyl group of the ACV valine, the third-residue side chain has sufficient freedom to move so as to let a water molecule into the sixth co-ordination site at iron (*trans* to Asp-216). Thus it appears that with its natural substrate, ACV, the enzyme induces a binding conformation in the isopropyl group that effectively 'reserves' this co-ordination site for dioxygen in a relatively hydrophobic environment. In this way the substrate facilitates dioxygen binding and thus promotes its own turnover

to the penicillin. This is further evidence that the active site of IPNS is exquisitely optimized to restrict (i.e. negatively catalyse [32]) all pathways except the one leading to the natural product IPN. Even a relatively small change in the hydrophobicity and steric requirements of the substrate upsets this balance and ultimately allows formation of products that would normally be disfavoured.

The presence of a water ligand in the oxygen-binding site of the IPNS-Fe(II)-ACAb complex also offers an explanation for the slower rate of ACAb turnover in solution studies with IPNS (relative to the natural substrate) [9]. It seems plausible that ACAb turnover should be hindered if a water ligand must first be displaced from iron before dioxygen can bind.

The structure of the IPNS-Fe(II)-ACAb-NO complex

The structure of the IPNS-Fe(II)-ACAb-NO complex was solved to 1.3 Å resolution from a crystal of the plate morphology (Figure 2, right-hand panel, and Table 1). The structure of the protein as a whole is essentially identical to those of previous structures [root mean square difference of 0.116 Å from the IPNS-Fe(II)-ACAb structure, for main-chain atoms] and the overall conformation of the substrate has changed little. Significantly, however, there is only one water ligand at iron: NO occupies the site *trans* to Asp-216 and has selectively expelled the water molecule from that site. The water ligand opposite His-214 remains (Wat-182 in the PDB deposition 1OBN), and there are the same number of water molecules in the vicinity of iron as in the IPNS-Fe(II)-ACV-NO complex. Although formation of the NO complex represents a small and rather subtle change in structure, the high resolution of the data made the high occupancy of the larger NO molecule clear in difference maps from the beginning of structure refinement.

The introduction of NO appears to displace the aminobutyrate γ -carbon from its partial occupancy of conformation II: the side-chain methyl group seems to exhibit complete occupancy of conformation I in the IPNS-Fe(II)-ACAb-NO complex. There is no evidence for any occupancy of conformations II or III, implying

Figure 4 Relative energies of the different D- α -aminobutyrate conformations at the active site of IPNS

The conformations corresponding to each of the positions (labelled **a–h**) are shown. Each torsion angle ($^{\circ}$) is shown with the His-270–Fe(II)–S plane vertical. Equivalent representations of the IPNS–Fe(II)–ACAb and IPNS–Fe(II)–ACAb–NO crystal structures are also shown for comparison (hydrogen atoms are included in these two representations for consistency, although the precise positions of these atoms are not resolved in either crystal structure). L-AA, L- δ -(α -aminoadipoyl).

near-complete occupancy of the ‘methyl up’ conformation I. [Note however that the absence of conformations II and III from the ground-state IPNS–Fe(II)–ACAb–NO complex does not preclude reaction via one of these conformations.]

Perturbation of the iron co-ordination chemistry upon substrate and NO binding has also been observed with several of the 2-oxoglutarate oxygenases, which are closely related to IPNS [33–36].

Modelling studies and mechanistic conclusions

It has been suggested that the three different products obtained from the incubation of ACAb with IPNS arise from different conformations of the ethyl group of a monocyclic intermediate [11,12]. Thus β -hydrogen abstraction from a monocyclic species which has the methyl group in conformation I is proposed to give the β -methyl penam, abstraction from the intermediate with the

γ -carbon in conformation II gives the cepham, and abstraction from the β -carbon when the side chain is in conformation III leads to the α -methyl penam.

Previous X-ray crystallographic evidence suggests that only small conformational changes are required in the region of the valinyl side chain during β -lactam closure from the IPNS-Fe(II)-ACV complex [3,16]. It may therefore be argued that the relative energies of the different aminobutyrate conformations in the IPNS-Fe(II)-ACAb-NO complex offer an approximation of the relative energies of corresponding monocyclic intermediates. Thus modelling calculations based on the IPNS-Fe(II)-ACAb-NO complex could provide some insight into the relative energies of side-chain conformations in the corresponding monocyclic intermediates (although absolute values for these energy differences clearly remain uncertain).

The two deepest energy minima in the plot of relative energy against conformation (Figure 4), **b** and **f**, correspond to structures in which the aminobutyrate γ -carbon is in conformations II and I respectively. The minimum at **b** has the γ -carbon in the space that is occupied by the valinyl pro-*R* methyl group in the IPNS-Fe(II)-ACV-NO complex, the position partially occupied in the IPNS-Fe(II)-ACAb crystal structure. In this orientation, one of the γ -hydrogens and the pro-*R* β -hydrogen atom are in close proximity to NO, and by analogy close to the putative iron(IV)-oxo moiety. Insertion of the high-valency species into each of these C-H bonds, followed by thiazolidine closure without rotation, would give rise respectively to the cepham **7** and the α -methylpenam **5**. The methyl position in minimum **f** (Figure 4) corresponds to the second of the two orientations seen in the IPNS-Fe(II)-ACAb complex, and to the only orientation that is occupied in the IPNS-Fe(II)-ACAb-NO crystal structure. It equates to a N-C α -C β -C γ torsion angle of 65.1°. In this conformation, the pro-*S* β -hydrogen atom is closest to the putative ferryl oxygen. Insertion into this C-H bond and thiazolidine closure would give the β -methyl penam **6**.

The small maximum that separates the minima at **b** and **f** includes positions **g**, **h** and **a** (Figure 4), all orientations in which the γ -carbon points towards the 'left' (as in conformation III, Figure 3). The unfavourability of conformation **a** results from steric clash of the methyl group with Ser-281, Leu-223 and Wat-251.

Positions **c**, **d** and **e** in Figure 4 correspond to conformations in which the aminobutyrate γ -carbon occupies positions that are intermediate between conformations I and II, pointing almost directly at the NO. In each of these positions, the major van der Waals interaction is between one of the γ -hydrogen atoms and the NO ligand. The energy of these conformations would presumably be lowered slightly upon formation of the ferryl intermediate since the reactive ferryl species [Fe(IV)=O $\leftrightarrow$ Fe(III)-O $\cdot$] should be less sterically demanding than diatomic NO.

Examination of the crystal structures and of the modelling results confirms that two of the three putative rotameric intermediates derived from ACAb are lower in energy than the third. There are only two clear energy minima, **b** and **f**, consistent with there being two clear methyl-binding pockets. Furthermore, it appears possible that all three observed products could be formed by oxo-insertion into the substrate while in one of these two orientations. (However given the approximate nature of the calculations, these findings do not rule out reaction via a higher energy conformation akin to conformation III.) The calculated minima **b** and **f** are equivalent to conformations II and I, and in essence they correspond to the binding pockets occupied by the methyl groups of the valinyl side-chain in the IPNS-Fe(II)-ACV complex (in which the β -hydrogen points away from iron). The two methyl groups in the anaerobic ACV complex sit 3.9 and

4.41 Å from iron [16], while the centres of the calculated binding pockets in **b** and **f** are each approx. 4 Å away from the metal. The isopropyl methyl groups must take up opposite positions in the monocyclic intermediate derived from ACV, to allow the rotation that brings the β -hydrogen towards the iron(IV)-oxo species [16]. This proposition is substantiated by the fact that the methyl groups in the IPNS-Fe(II)-IPN complex [3] occupy approximately the same regions of space as in the IPNS-Fe(II)-ACV-NO structure [16].

Since this modelling study only takes into account van der Waals radii and does not include consideration of the changes that occur in formation of the monocyclic intermediate, it would be imprudent to read too much into the calculations. Nonetheless, it is interesting that the two minima **f** and **b** correspond to conformations from which the major products **6** and **7** are thought to arise. Furthermore, the modelling results suggest that the α -methyl penam **5** could also be formed from reaction in conformation II. Alternatively, if formation of **5** does require reaction via conformation III, the higher energy of **a** is consistent with observations that the α -methyl penam is isolated only in trace amounts.

Conclusions

These results reveal how subtle conformational changes are involved in determining the product selectivity of IPNS, particularly in its reaction with 'unnatural' substrates. A combined crystallographic and molecular modelling approach aids in the rationalization of previous observations made of the enzyme in solution. This sort of approach is also likely to be applicable to the study of related oxygenases such as deacetoxycephalosporin C/deacetylcephalosporin C synthase ('DAOC/DACS') where conformational changes during catalysis have also been invoked as inherent to selectivity [37,38].

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