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Structure of a mimic of aminoacylated tRNA determined by means of transferred NOE data

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ABSTRACT: Anthraniloyladenosine (Ant-Ado) serves as an analog for aminoacylated tRNA and is specifically recognized by bacterial elongation factor Tu (EF-Tu). In this compound, adenosine is esterified with anthranilic acid, which mimics an α-amino acid. The weak binding of Ant-Ado to the EF-Tu gives rise to transfer-NOE (TrNOE) signals from which the conformation of Ant-Ado in its protein-bound state can be deduced. To this end a full relaxation matrix approach was used with the *R*-factor as a target function. The position of the base was determined by a mapping procedure. Structures of ribose and anthranilic acid were obtained by simulated annealing. The nucleotide was found to be in 2′-*endo* puckering, with the base in an *anti* conformation. The anthranilic acid adopts a position in which an amino group is presented in a similar way as by phenylalanine in the crystal structure of the ternary complex EF-Tu · GPPNHP · Phe-tRNA^{Phe}. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: NMR; ^{1}H NMR; transferred NOE; nucleotide structure; molecular recognition

INTRODUCTION

In the course of bacterial protein biosynthesis, an important recognition step is accomplished by the elongation factor Tu (EF-Tu). This protein serves to distinguish between aminoacylated (charged) and non-aminoacylated (uncharged) tRNAs. Since both the individual tRNA molecules and the 20 natural amino acids differ appreciably, the recognition element must be located in a region common to all tRNAs. Such a domain is given by the ubiquitous 3′-CCA end of tRNA, which is aminoacylated at the 3′-terminal nucleotide, adenosine-76.[1]

Previous studies had shown that a single adenosine is bound by the EF-Tu if it is aminoacylated with phenylalanine.[2,3] However, phenylalanyladenosine turned out not to be a favorable ligand for detailed binding studies owing to its relatively fast deacylation. We therefore used the slowly hydrolyzing analog anthraniloyladenosine (Ant-Ado) (Fig. 1). In this compound, the adenosine is esterified not to an α-amino acid, but to anthranilic acid, which hydrolyzes much more slowly owing to conjugation of the ester group with the aromatic system. That anthranilic acid is suitable as an amino acid analog has been shown before using anthranilated tRNA^{Phe}, which has a similar affinity to EF-Tu · GTP as native phenylalanylated tRNA^{Phe}.[4]

In two previous papers we have presented the solution conformation and crystal structure of free Ant-Ado[5] and binding characteristics of Ant-Ado to EF-Tu and its conformation in the enzyme-bound form.[6] The 3′-isomer was found to be bound much more strongly than could be derived from the degree of line broadening. Free in solution, the ribose of 3′-Ant-Ado is puckered 2′-*endo* and the adenine moiety adopts an *anti* conformation.[5] When bound to the EF-Tu, the ribose proton signals of 3′-Ant-Ado broaden most strongly, indicating an involvement of the 2′-OH group in the binding process.[6]

Information on the conformation in the bound state originates from the observation of transfer-NOEs which are a consequence of the different rotational correlation times for the small ligand molecule and the large protein–ligand complex, in combination with fast

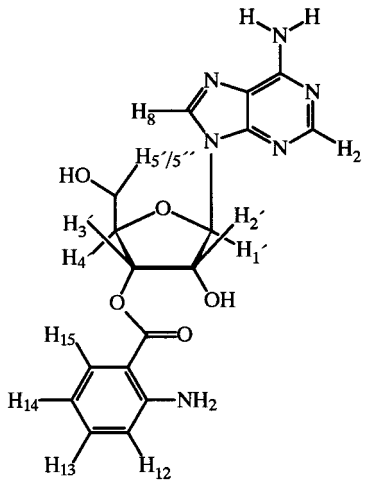

Figure 1. Structure of 3′-anthraniloyladenosine (3′-Ant-Ado). The proton numbering used in the text and in Table 1 is indicated.

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exchange on the NMR time-scale between free and enzyme-bound forms of the ligand.[7] It requires the ligand to be bound not very tightly (i.e. in the range 10^{-3}–10^{-6} M)[8] to the protein, which condition is fulfilled for the binding of Ant-Ado to EF-Tu, in contrast to the aminoacylated tRNA.[6] We have already presented a conformational model based on the transfer-NOE data.[6] Here, we outline this structure analysis in greater detail.

EXPERIMENTAL

Acquisition and analysis of NMR data

The preparation of the NMR samples has been described before.[6] Briefly, 1 mM solutions of anthraniloyladenosine in 7:1 D_2O–methanol-d_4 were used. Under these conditions the protein retains its biological activity, which is demonstrated by the fact that binding of intact aminoacylated tRNA and GTP hydrolysis are essentially unaffected (B. Nawrot and N. Grillenbeck, personal communications). For the structure determination, generally protein concentrations of 0.05 mM were employed.

All NMR spectra were acquired at 500 MHz on a Bruker DRX 500 spectrometer equipped with an inverse broadband probehead. ^{1}H NOESY spectra were acquired phase sensitive in t_1 [9] with 4K data points in t_2 and 512 t_1 increments and a 10 ppm spectral width in both dimensions. ZQ-COSY peaks were suppressed by the method of Rance *et al.*[10] Mixing times of 70 and 150 ms were employed with a 2 s recycle delay. Water suppression was achieved by presaturation.

For data analysis, the NDEE program package (Software Symbiose, Bayreuth, Germany) was used. Two-dimensional spectra were apodized by multiplication with a 90° phase-shifted squared sine-bell function before Fourier transformation. All shifts were referenced to the residual methanol signal (3.5 ppm). Cross peak volumes were determined with the integration routine of the NDEE program.

Structure calculation

For the structure calculation, all measured NOE intensities were assumed to originate solely from the transfer-NOE. The portion of the intensity due to the free ligand (which is negative and thus leads to an attenuation of the signals) was neglected. Under the conditions employed in our study, the NOE cross peaks are at least one order of magnitude smaller than the transfer-NOEs.[5]

Structures were calculated with the Xplor program package, version 3.851. An RNA-specific force field[11] was used for the nucleotide, whereas the force field parameters for bonds involving protons and the anthraniloyl moiety were adapted from the CHARMM forcefield. In all cases, the *R*-factor,[12] $R = \Sigma(I_{calc} - I_{obs})^n/\Sigma I_{obs}^n$, with $n = 1/6$, served as a criterion for assessing the agreement between calculation and experiment. Simulated annealing with the full relaxation matrix[13,14] was achieved by cooling the structure from 1200 to 50 K within 1 ps. Rotational correlation times and mixing times were optimized as described under Results. Exchangeable amino and hydroxy protons were assumed to be fully deuterated. All calculations (see Results) were repeated in an iterative manner.

RESULTS

Transfer-NOEs for 3′-Ant-Ado in the complex

EF-Tu is a GTP/GDP binding protein and possesses an intrinsic GTPase activity.[15] It is therefore complexed with a guanosine nucleotide (GTP or GDP). The affinity of EF-Tu · GTP for tRNA is several orders of magnitudes higher than that of EF-Tu · GDP,[16,17] which can be easily understood in terms of structural reorganizations of the protein in the course of GTP hydrolysis.[18–20] Consequently, we measured transfer-NOEs of the ternary complex EF-Tu · GTP · Ant-Ado. To circumvent GTP hydrolysis (and the structural rearrangements) during the NOESY measurement, all these measurements had to be carried out at low temperature.

In Fig. 2, the region of the resulting NOESY spectrum containing NOEs to the H-8 protons of both isomers is shown. The main problem is the overlap of

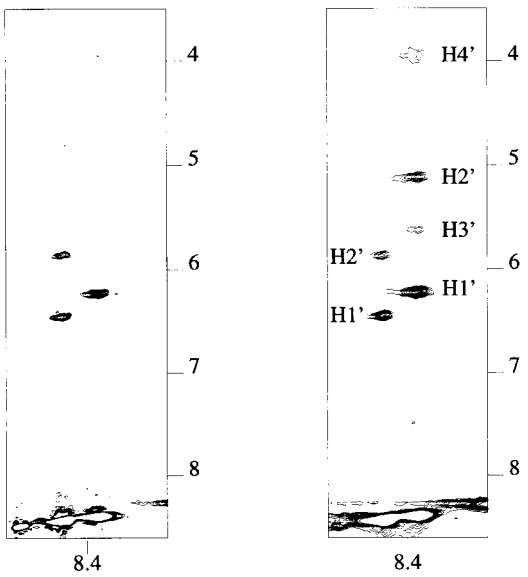

Figure 2. Portions of the NOESY spectrum displaying NOEs involving the adenine H-8 proton. The diagonal can be seen in the bottom part of the spectrum. The signals of the two isomers 2′-Ant-Ado (8.409 ppm) and 3′-Ant-Ado (8.356 ppm) can be distinguished. Left, spectrum at 281 K (complex with EF-Tu · GTP); right, spectrum at 298 K (complex with EF-Tu · GPPNHP). At 281 K, partial bleaching out of the signals from 3′-Ant-Ado due to overlap of the 3′-proton with the water signal is observed. For the latter spectrum, the assignment of the cross peaks is indicated.

Table 1. Cross peak intensities for transfer-NOE of 3′-Ant-Ado in complex with EF-Tu · GPPNHP at 298 K[a]

		70 ms	150 ms
H-8	H-1′	17.6	125.0
H-8	H-2′	10.6	86.0
H-8	H-3′		21.0
H-8	H-4′		6.8
H-8	H-5′/H-5″		93.3
H-1′	H-2′		37.6
H-1′	H-3′		9.6
H-1′	H-4′		9.2
H-1′	H-5′/H-5″		33.1
H-1′	H-14		1.5
H-1′	H-15		4.3
H-2′	H-3′	26.3	94.0
H-2′	H-4′	2.1	21.3
H-2′	H-5′/H-5″	67.7	105.0
H-3′	H-4′	1.1	39.3
H-3′	H-5′/H-5″	38.7	173.0
H-4′	H-15		6.8
H-12	H-13	80.0	230.0
H-12	H-15		18.0
H-13	H-14	80.0	230.0
H-13	H-15		29.0
H-14	H-15	49.0	300.0

[a] Numbers indicate NOEs between the protons in the first and the second columns at two different mixing times. All intensities are given in arbitrary units.

the water signal and the 2′-proton of the 3′-isomer. This leads to partial saturation of all protons in this isomer due to extensive spin diffusion (Fig. 2, left), which means that cross peak intensities cannot be determined correctly and that a structure determination is not possible.

We therefore used EF-Tu complexed with the non-hydrolyzable GTP analog GPPNHP. This enabled us to work at higher temperatures (25 °C) where the water line is shifted far enough upfield to prevent overlap with

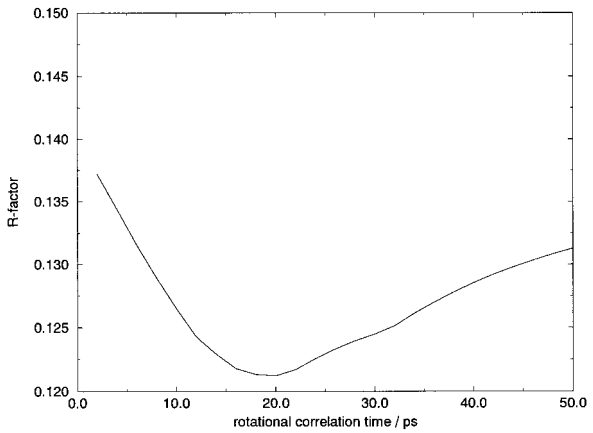

Figure 3. Dependence of the *R*-factor on the rotational correlation time assumed in the calculation. A minimum at *ca*. 20 ns can be observed, which indicates good agreement between experiment and calculation, and agrees well with the value expected for a protein with the size of EF-Tu (45 kDa).

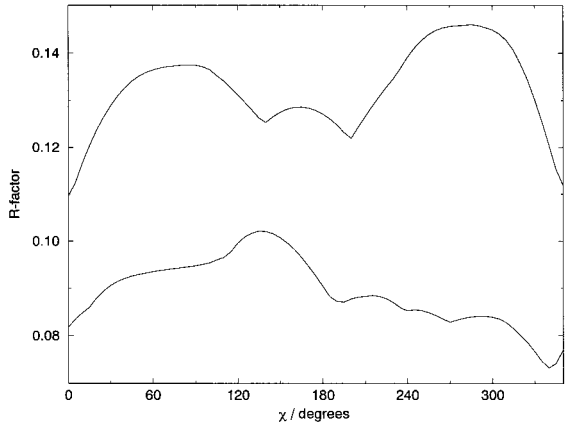

Figure 4. Mapping of the dihedral angle χ, describing the orientation of the base with respect to the ribose ring. The curves show the compatibility with the experimental data for all values of χ for mixing times of 70 ms (upper trace), and 150 ms (lower trace). In addition to the global minimum at 350°, a second minimum at 190° can be observed.

any proton resonances of the 3′-isomer. The resulting spectrum is displayed on the right of Fig. 2. The number and intensity of NOEs to the H-8 proton increase dramatically.

Altogether, 22 NOEs were detected at 150 ms and 10 NOEs at 70 ms mixing time. In Table 1, the corresponding cross peak intensities are compiled. Owing to extensive spin diffusion, the intensities are levelled out at the longer mixing time of 150 ms. Consequently, structure determination with the isolated spin pair approximation (ISPA), taking into account the fixed intra-aromatic NOEs in conjunction with data from the x-ray structure analysis of 3′-Ant-Ado[5] as a reference, led to results which were geometrically not possible.

Relaxation matrix refinement: optimization of parameters

Owing to the failure of the ISPA approach, the structure had to be determined by quantitative calculation of the relaxation matrix. A number of parameters must be known to enable such a calculation to be performed. The most important one is the rotational correlation time, which determines the efficiency of the different relaxation pathways, and thus directly influences the NOE intensities. A typical plot of the *R*-factor as a function of the rotational correlation time is depicted in Fig. 3. The curve has a minimum at a rotational correlation time of about 20 ns, which agrees excellently with the value to be expected for a protein with the size of EF-Tu (45 kDa).[21]

A similar calculation can be performed regarding the optimization of the mixing time used in the calculation, taking into account that ligands are present in the protein-bound form during a fraction only of the experimental mixing time. In this case, optimum theoretical

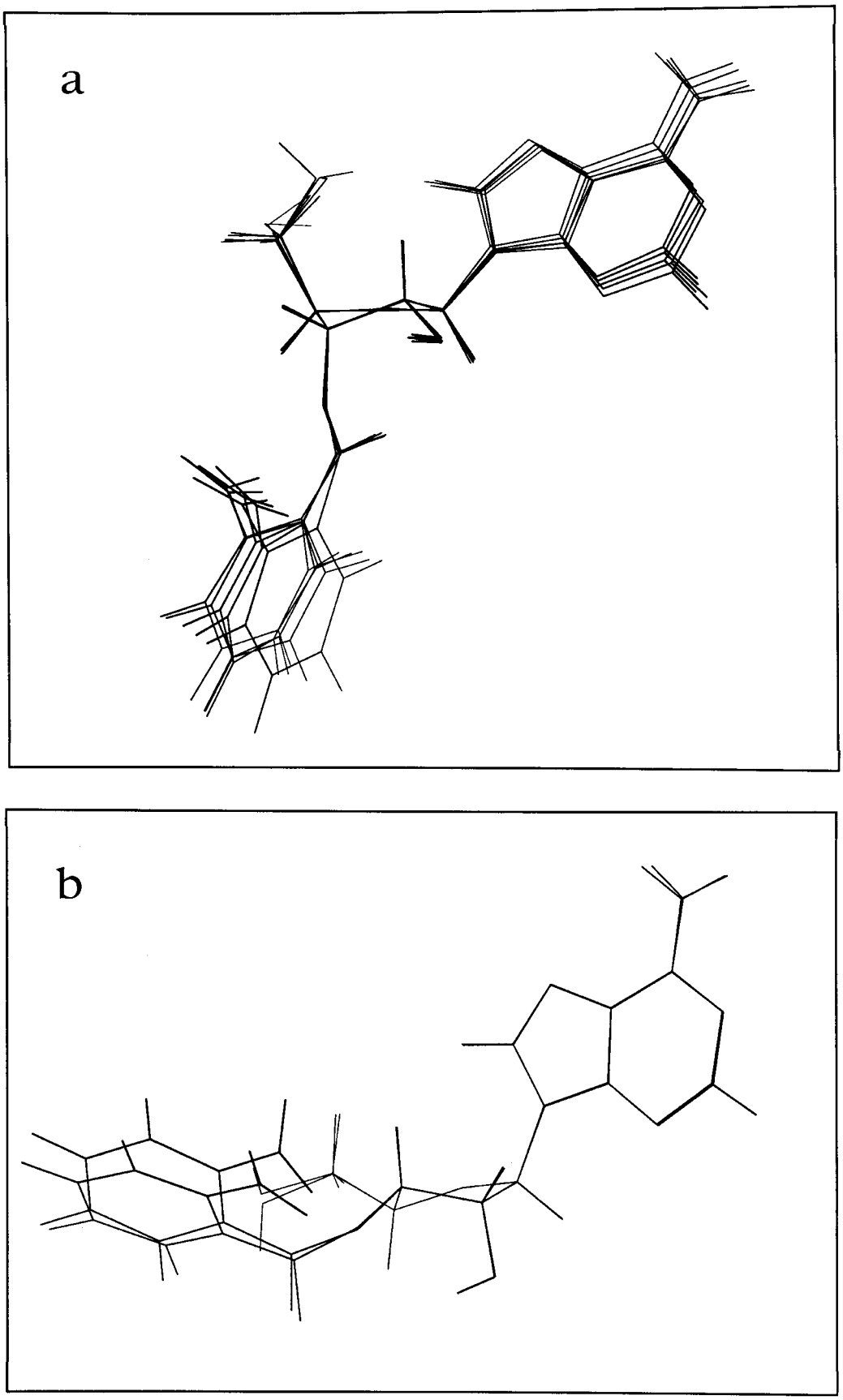

Figure 5. (a) Five structures with low *R*-factors, apparently representing the best agreement with the NOE data. The amino group is oriented towards the viewer while the carbonyl group of the ester linkage points in the opposite direction. The ribose ring was used for superposition of the molecules. (b) Two structures with high *R*-factors, with 3′-*endo* conformation of the ribose presenting the amino and the carbonyl groups in very different ways compared with the structures shown in (a).

mixing times were determined which were *ca.* 20% shorter than the experimental values (data not shown).

By contrast, all attempts to take into account either T_1 leakage effects or different order parameters in the ligand did not lead to any meaningful results. Therefore, both effects were neglected.

Determination of χ

Ant-Ado consists of three units, namely the adenine base, the ribose and the anthraniloyl residue, the relative orientations of which are to be determined. The small number of protons on the adenine moiety is balanced by the fact that the orientation of the base relative to the ribose is determined by a single parameter, the dihedral angle χ (O-4′—C-1′—N-9—C-4). Figure 4 shows the *R*-factor for all possible values of χ. The two curves represent data from the two mixing times used in the calculation. Both plots have in common the global minimum at *ca.* $-10°$ (e.g. *syn* conformation) and a local minimum at 200° (*anti* conformation). Since, however, a *syn* conformation would give rise to very strong NOEs between the adenine H-2 proton and the ribose protons, which were not detectable at all, the local minimum corresponding to an *anti* conformation is obviously the correct one. Furthermore, the global minimum around 0° appears not to reflect the real situation as such a value has never found before for the dihedral angle χ,[22] whereas the value expected for a normal *syn* conformation lies at 80°. In contrast, the values of the local minimum agree well with those found for *anti* conformation.

Structure calculation for the ribose and anthraniloyl residue

Neglecting the orientation of the 5′-OH group, which cannot be determined in our case because the H-5′/H-5″ protons resonate at the same frequency, the only conformational degree of freedom of the ribose is the ring puckering. By contrast, the orientation of the anthraniloyl moiety relative to the ribose is determined by no less than three torsional angles, and likewise by the ribose pucker. Consequently, all values were refined simultaneously with the optimum value found before for χ kept fixed.

The outcome of these calculations is displayed in Fig. 5. Figure 5(a) shows the structures with the lowest *R*-factors (*ca.* 0.1). All these structures exhibit fairly good agreement. The ribose is puckered 2′-*endo* and the anthraniloyl ring is positioned 'below' the ribose. This is in good agreement with the observation of three weak NOEs between ribose H-1′ and H-4′ protons and base protons of the anthraniloyl ring (cf. Table 1), which have distances between 3.9 and 6 Å, respectively, in these structures. Indeed, a quantitative evaluation of NOE intensities yielded excellent agreement between the predicted and measured NOEs for these connectivities, while the deviations were much larger for the stronger NOEs.

Figure 5(b) shows two structures with the highest *R*-factors (*ca.* 0.13). These have very different conformations. The ribose is now puckered 3′-*endo*, which brings the anthraniloyl ring at a far distance from the ribose. The respective distances corresponding to the above-mentioned NOEs are now in the range of up to 8 Å. Indeed, this orientation of the anthraniloyl ring relative to the ribose or similar structures represent the only arrangements where these NOEs are expected. Hence the orientation of the amino and the carbonyl groups is also fixed.

DISCUSSION

Structure calculation

The high residual *R*-factors for the resulting structures point to inaccuracies in the structure determination scheme employed here. However, this is not unexpected: only data for two NOESY mixing times were used in the calculations, and no quantitative evaluation of NOE build-up curves was carried out. Furthermore, the relaxation matrix neglects all protein protons. In particular, the binding pocket for the adenine base contains several proton-rich, hydrophobic amino acid side-chains (Val237, Ile231 and Leu289).[20] These protons can probably account for the discrepancies between back-calculated and experimental NOEs. The former were found to be higher than the latter, which is explainable by mediating protein spins. In contrast, for other conceivable error sources (underestimation of the spin diffusion, neglect of NOEs from the 'free' ligand) the opposite behavior is expected.

While a more elaborate calculation utilizing the coordinates of the crystal structure[20] for the ternary complex EF-Tu · GPPNHP · Phe-tRNA^{Phe} might yield a higher precision, the available data can consistently and reliably be interpreted in terms of a meaningful structure.

Structure of the nucleotide

The nucleotide part of Ant-Ado adopts a structure not very different from the solution structure of the free ligand,[5] which is also consistent with the structure of aminoacylated tRNA in the ternary complex EF-Tu · GPPNHP · Phe-tRNA^{Phe}.[20] The conformation of the ribose was found to be 2′-*endo* in both cases. This would lead to destacking above a 5′-adjacent nucleotide, which is also observed in the previously mentioned crystal structure.[20] One limitation of the present study is the smallness of the single nucleotide, which yields no information concerning the stacking behavior. Consequently, other small analogs such as CA-Ant or CCA-

Ant, both simulating the aminoacylated CCA end of tRNA while maintaining the smallness required for observation of transfer-NOEs, are the logical next step in structure determination.

The nucleotide structure is thus in a range which is not unexpected for RNA. This confirms that the resulting structure is fairly reliable.

Position of the anthraniloyl residue

The linkage between the α-amino acid or the anthraniloyl residue contains the functional groups common to all α-amino acids which constitute the recognition element for the EF-Tu. Consequently, the positioning of the two functional groups which were found to be important for tRNA recognition in the crystal structure,[20] namely the amino and the carbonyl groups, is important. In this crystal structure, the amino residue is presented in a similar orientation to that found in the TrNOE structure (Fig. 5). By contrast, the carbonyl group is pointing in a very different direction. It is improbable that a complete coincidence for both groups can be obtained with 3′-Ant-Ado since the relative positions of the two are not identical with those adopted in an α-amino acid. This shows the limits of an analog for detailed structural binding studies. In the crystal structure, the carbonyl oxygen is hydrogen bonded to Arg274, which is not possible in the TrNOE structure of 3′-Ant-Ado. It is conceivable, however, that other functional groups of the protein are bound to this oxygen.

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