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# Effect of protein side chain amide group on the hydrogen-bond equilibrium in nucleobases studied by infrared and $^{13}$C-NMR spectroscopy

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Infrared spectra of 1:1 hydrogen-bonded complexes formed by derivatives of adenine and model molecules bearing the protein side chain amide group have been measured in chloroform solution. From the temperature dependence of hydrogen-bond formation, thermodynamic data on these complexes are determined. On the basis of these data, it is shown that the complexes consist of cyclic heterodimers, those that use the adenine N(1)H bond being favoured. Similarly, infrared and $^{13}$C-NMR spectroscopy reveals that uracil-amide cyclic heterodimers formed through the uracil 4-carbonyl group are predominant. All of these results predict that Watson-Crick hydrogen bonds in adenine-uracil base-pairs may be opened to some extent, as proved in this work. The possible biological importance of these observations is also discussed.

## 1. Introduction

With only a few exceptions, naturally occurring DNAs are fully double-stranded helices formed from two complementary single strands. However, to preserve the information stored in DNA by replication, or to read out that information by transcription, it appears necessary to form locally single-stranded regions by protein-mediated physical disruption of the double helix. Experiments that probe the characteristics of the interconversion of DNA double- and single-stranded conformations are thus of central biological importance. A large proportion of the forces holding nucleic base-pairs together in polynucleotide helices comes from hydrogen bonding. Hence, the separation of the two strands in the presence of helix-destabilizing proteins must comprise the dissociation of at least part of these hydrogen bonds. This helix opening may be partly due to the formation of other hydrogen bonds between the protein hydrophilic groups and the Watson-Crick binding sites. Therefore, it appears worthwhile to study the way in which the hydrogen-bond equilibrium in nucleic bases is affected by the polar groups in protein side chains, in the hope that different hydrogen bonds can be selectively formed. This selectivity in hydrogen bonding may also help to explain the recognition of nucleic acid base pairs by protein side chain groups. Some works [1–3] have been devoted to the infrared study of the interactions between derivatives of adenine or uracil and compounds bearing hydroxyl or carboxyl groups. We report here a thermodynamic and structural investigation of hydrogen-bond interactions between adenine or uracil bases and model molecules bearing the protein side-chain amide group using infrared and $^{13}$C-NMR spectroscopy.

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## 2. Experimental

### 2.1. Materials

9-Ethyladenine (A) and 1-cyclohexyluracil (U) were purchased from Sigma. The amide derivatives, acetamide and caprolactam, were obtained from Merck. All these substances were dried under vacuum, kept in a refrigerator and used without further purification. $C^2HCl_3$ was used as solvent (from Merck; Uvasol).

### 2.2. Methods

Infrared spectra were recorded on a Perkin-Elmer model 599B infrared spectrophotometer and were acquired using an on-line microcomputer at 1.0 $cm^{-1}$ intervals. This spectrophotometer was provided with a VLT-2 variable-temperature cell (Research and Industrial Instruments, London, U.K.). The spectra were digitized and transferred to the CDC Cyber 180/855 of the CSIC, Madrid, for band evaluation. In this connection, a fitting program for resolution of the spectral profiles was used [4]. Following band separation, thermodynamic data on hydrogen-bond formation in adenine heterodimers were determined by means of the method of Kyogoku et al. [5] for equimolar solutions of A and caprolactam over the concentration range 0.1–0.02 M.

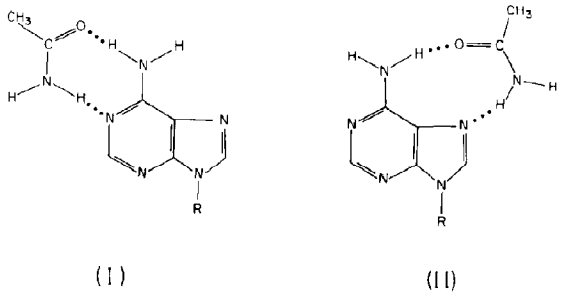
The $^{13}C$-NMR spectra were recorded by means of a Bruker WM-360 spectrometer operating at 90 MHz in the Fourier transform mode. The chemical shifts reported are expressed in $\delta$ (ppm) downfield from $Me_4Si$.

In order to evaluate the association constants for the amide ligand (L) at the N1 and N7 purine sites, prior estimation of the trimer association constant ($K_t$) is required for the following equilibria in mixed solutions containing A, U and L:

$$AU + L \rightleftharpoons AUL \quad (1)$$

$$AL + L \rightleftharpoons AL_2 \quad (2)$$

If it is assumed that there is no interaction between the N1 and N7 binding sites of adenine, where cyclic heterodimers can be formed (fig. 1),



(I) (II)

Fig. 1. Structures of dimers between A and acetamide.

$K_t$ must be equal for both equilibria and is defined as follows:

$$K_t = (C_{AUL} + C_{AL_2})/(C_{AU} + C_{AL})C_L \quad (3)$$

where $C_L$ represents the concentration of the L monomers, $C_{AL}$, $C_{AU}$, $C_{AUL}$ and $C_{AL_2}$ corresponding to those of their respective hydrogen-bonded dimers and trimers. The sum $C_{AU} + C_{AL}$ can be estimated from the absorbance of the 3485 $cm^{-1}$ band of adenine (figs. 3 and 4). It is assumed that the molar extinction coefficient at this frequency is the same for both AU and AL heterodimers, as this band is due to the stretching vibration of the nonbonded NH bond in the purine $-NH_2$ group (fig. 1). After using the fitting program for band resolution, $C_A$, $C_L$ and the absorbance at 3485 $cm^{-1}$ ($A_{3485}$) can be measured. Moreover, the association constants $K_{AA} = C_{AA}/C_AC_A$, $K_{AU_2} = C_{AU_2}/C_{AU}C_U$, $K_{AU} = C_{AU}/C_AC_U$, $K_{AL} = C_{AL}/C_AC_L$ and the equation $A_{3485} = \epsilon bC_{AU} + \epsilon bC_{AL}$ enable us to determine $C_{AU_2}$, $C_{AU}$, $C_{AA}$ and $C_U$. $\epsilon$ and $b$ denote the molar extinction coefficient of the 3485 $cm^{-1}$ band and the cell thickness, respectively. In these equations, we employed the $K_{AA}$, $K_{AU}$ and $K_{AU_2}$ values reported in the literature [5,6]. $K_{AL}$ has been determined in this work as described above. On this basis and taking $C_A^o$ as the total concentration of 9-ethyladenine, we obtain $C_{AUL} + C_{AL_2}$ from the following relation:

$$C_{AUL} + C_{AL_2} = C_A^o - C_A - 2C_{AA} - C_{AL} - C_{AU} - C_{AU_2} \quad (4)$$

From the values of $C_{AUL} + C_{AL_2}$, $C_{AU} + C_{AL}$, and $C_L$, the association constant $K_t$ can then be estimated by applying eq. 3.

## 3. Results and discussion

### 3.1. 9-Ethyladenine / amide association

Fig. 2 includes the infrared NH stretching region of an equimolar mixture of the adenine derivative and acetamide. Comparison of this spectrum with that of the sum of the noninteracting compounds (fig. 2) leads to the following observations. The relative intensities of the bands near 3535 $cm^{-1}$ ($\nu_{as}NH_2$) and 3417 $cm^{-1}$ ($\nu_sNH_2$) of monomers are decreased, while the absorption in the 3400–3200 $cm^{-1}$ region increases due to heteroassociation. When A or acetamide forms a dimer through the amino group, one of the two NH bonds is bonded with the partner molecule and the other is free. The stretching frequency of this nonbonded NH ($\nu_fNH$) is expected to appear between the $\nu_{as}NH_2$ and $\nu_sNH_2$ bands of the free amino group. Heteroassociation causes the appearance of two $\nu_fNH$ bands at 3498 and 3485 $cm^{-1}$ arising from acetamide and A, respectively (fig. 2). The 3498 $cm^{-1}$ $\nu_fNH$ frequency of acetamide is characteristic of the cyclic heteroassociation of this substance [7]. Therefore, 1:1 cyclic complexes appear to predominate in the equimolar mixed solutions of A and acetamide over the concentration range used in this work. All sites of hydrogen-bonding interactions cannot be available during the recognition of nucleic acids by proteins. It is therefore important to determine the association constants for the various 1:1 cyclic complexes (fig. 1). The infrared spectroscopic methods of determining the strength of hydrogen bonding in heterodimers involve absorbance measurements for the bands corresponding to the nonbonded polar groups in monomers [5]. As the $\nu_{as}NH_2$ and $\nu_sNH_2$ bands of A and acetamide monomers are practically coincident, such absorbance measurements would be unreliable in the case of A-acetamide heteroassociation. It is therefore convenient in these measurements to replace acetamide by another substance having an amide group in the *cis* configuration, which is in fact the one that is able to form cyclic heterodimers with A. Caprolactam possesses this property, apart from having proton-accepting and proton-donating capacities similar to those of acetamide [8–10].

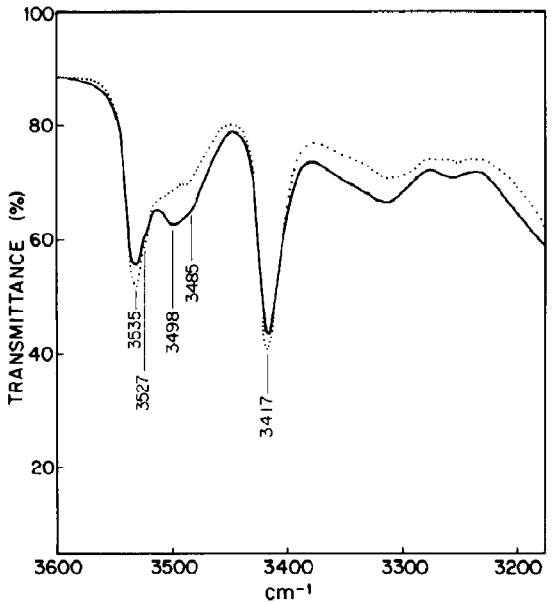



Fig. 2. (———) Infrared spectrum of an equimolar mixture of A and acetamide (0.12 M each). (· · · · · ·) Sum of the spectra of separated components. (0.1 mm KBr cell).

When equimolar solutions of A and caprolactam are mixed together, spectral changes occur with respect to the sum of the spectra of the separate components (fig. 3). Thus, the $\nu_fNH$ band of the amino group in A (3485 $cm^{-1}$) and the bonded NH stretching band (3320 $cm^{-1}$) increase in strength at the expense of a reduction in the relative intensities of the free NH stretching bands at 3527 and 3419 $cm^{-1}$. The 3527 $cm^{-1}$ band is due to the $\nu_{as}NH_2$ mode of the A monomer, and the absorption maximum at 3419 $cm^{-1}$ results from masking of the 3417 $cm^{-1}$ band of the A monomer by the 3419 $cm^{-1}$ band of the free NH stretching mode of caprolactam.

The above spectroscopic results appear to contrast with those obtained by others [1,11], who stated that the mixing of A or U with acetamide or valerolactam does not induce modification of the infrared spectra that can explain the interaction between these amides and nucleobases. However, this apparent conflict arises from the fact that the concentrations used in this work (figs. 2–4 and 6) were about 3.0–20.0-times greater than those used in the previous investigation [1,11].

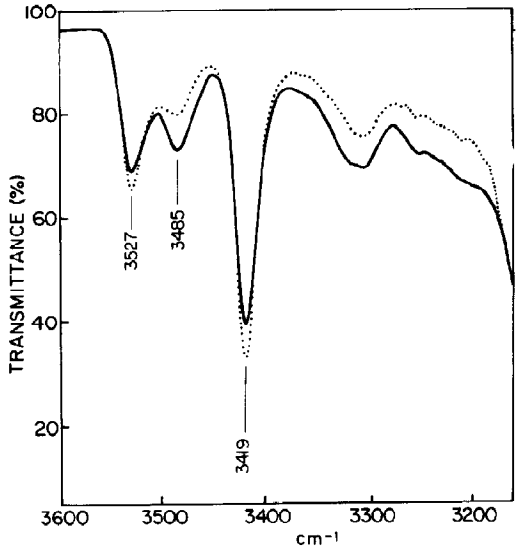



Fig. 3. (———) Infrared spectrum of an equimolar mixture of A and caprolactam (0.1 M each ). (· · · · · ·) Sum of the spectra of separated components. (0.2 mm NaCl cell).

Indeed, we have verified in our laboratory that clearly evident modifications in spectra of the mixed solutions of the amides and nucleobases are not observable using the concentrations reported in those earlier works.

We have determined thermodynamic data for hydrogen-bond formation between A and caprolactam species taking into account absorbance measurements of the A monomer at 3527 $cm^{-1}$. As this band overlaps with that of $\nu_f NH$ at 3485 $cm^{-1}$ (fig. 3) absorbance measurements were carried out after band separation. The method of Kyogoku et al. [5] has been applied to the A-caprolactam system by assuming that cyclic dimers predominate in the mixed solution. In support of this assumption, we cite the following fact. The concentration of A relative to that of caprolactam remains practically constant (1.0) over the concentration range considered in this work, as shown by the relative intensities of the $\nu_{as}NH_2$ and $\nu NH$ bands of A and caprolactam monomers, respectively. This demonstrates that the amino group of A and the imino group of caprolactam are involved in heterodimer formation at a constant ratio, which supposes that the heterodimer must be cyclic. Another way of distinguishing between the open and cyclic forms is to measure $\Delta H^o$ for the association of a dimer, which should be about twice as large for the cyclic dimer as for the open dimer [12,13]. To obtain thermodynamic data for the A-caprolactam system we recorded the spectra as a function of the temperature of the mixed solution. $\Delta H^0$ and $K_{AL}$ were found to be $-5.7 \pm 0.5$ kcal/mol and $18.2 \pm 1$ $M^{-1}$ from a van't Hoff plot of the association equilibrium. The value of the enthalpy is about 2-fold greater than the heat of formation of a single NH...O=C hydrogen bond as measured in chloroform [12] and confirms the existence of cyclic dimers in solution. Since alternative A-amide cyclic arrangements may be possible (fig. 1) which have interesting biological implications, we felt that an examination of the relative chemical affinity of A for the *cis* part of the amide group was warranted. One approach to resolving this problem involves evaluation of the formation constants of A-containing heterotrimers in solution and comparison of the values with those predicted on a statistical

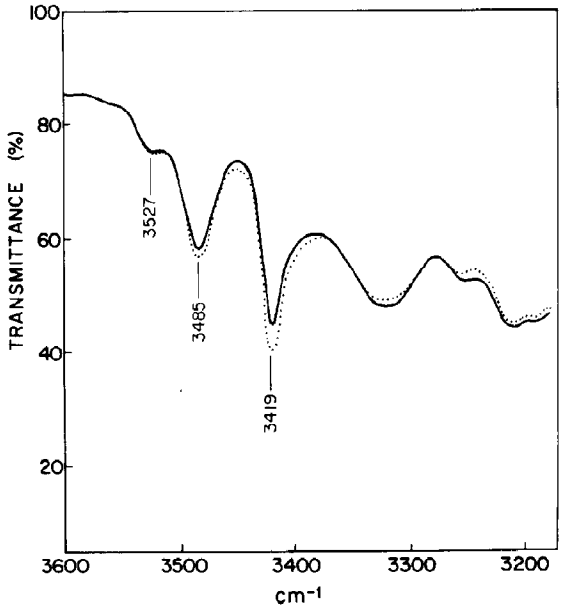



Fig. 4. (———) Infrared spectrum of a mixed solution containing 0.16 M A, 0.16 M U and 0.24 M caprolactam. (· · · · · ·) Sum of the spectra of separated components. (0.1 mm KBr cell).

basis. In this connection, the addition of excess caprolactam to an equimolar, dilute solution of A and U will result in the appearance of cyclic heterotrimers some of which contain molecules of both A and caprolactam. In fact, the separation of bands in the spectra of these solutions reveals that the relative intensity of the 3485 $cm^{-1}$ $\nu_f$NH band of the bonded A in AU base-pairs decreases upon addition of caprolactam (fig. 4).

Let the subscripts 1 and 7 represent the above-mentioned binding positions in adenine. The association constant measured as described above, $K_{AL}$, is:

$$K_{AL} = C_{AL}/C_A C_L = (C_{A1} + C_{A7})/C_A C_L = k_1 + k_7 \quad (5)$$

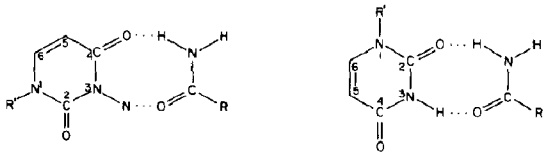
$k_1$ and $k_7$ being the association constants for the attachment of the *cis* amide group through the pyrimidine (I) and imidazole (II) rings, respectively (fig. 1). Conversely, let $C_{AX}$ represent the sum $C_{AL} + C_{AU}$, X corresponding obviously to U or lactam. Eq. 3 then leads to:

$$\begin{aligned} 1/K_t &= C_{AX} C_L/(C_{AUL} + C_{AL_2}) \\ &= (C_{A1X} + C_{A7X}) C_L/(C_{AUL} + C_{AL_2}) \\ &= 1/k_1 + 1/k_7 \end{aligned} \quad (6)$$

If $K_t$ is known, $k_1$ and $k_7$ may be estimated from eqs. 5 and 6. Their respective values were found to be $k_1 = 12.4 \pm 1.3$ $M^{-1}$ and $k_7 = 5.8 \pm 0.5$ $M^{-1}$. The fact that $k_1$ is about 2-fold greater than $k_7$ can be explained as follows. The geometry of the complexes (fig. 1) is such that O...H–N hydrogen bonds are linear for a complex of type I and slightly bent for type II. Another cause of the ratio $k_1/k_7$ may be the difference in basicity between the N1 and N7 binding sites [14,15]. Irrespective of the origin of this difference in binding strength, the $k_1/k_7$ ratio predicts a decrease in melting temperature ($T_m$) of the poly(A) · poly(U) helix in the presence of the protein side-chain amide group in aqueous solution. Earlier results in our laboratory [16] support this conclusion. This lowering of $T_m$ may result from stronger binding of the amide group on the N1 adenine sites of single-stranded poly(A) combined with weaker binding to the N7 adenine sites of the poly(A) · poly(U) helix.The fact that the specificity of hydrogen bonding in chloroform solution can exist in naturally occurring polynucleotides may not be entirely fortuitous, since the interior of the helix and the more immediate environment of nucleobases are also nonaqueous, with the hydrogen bonds of the base-pairs being enclosed in a domain of the stacked unsaturated rings [5,17]. Some experiments on the countercurrent distribution of DNA from bacteria [17,18] showed that newly replicated DNA is more soluble in a nonaqueous phase than in an aqueous one. All this suggests that interactions in nonaqueous solvents may have more relevance to the behaviour in biological systems than would be anticipated otherwise. Therefore, the results on the thermodynamics in chloroform cannot simply be extrapolated to the respective interactions in aqueous solution, however, the relative values of the association constants may be similar in both environments whereby comparable selectivity in hydrogen-bond formation may occur [3,5].

### 3.2. 1-Cyclohexyluracil / amide association

Interaction between U and acetamide leads to the formation of 1:1 cyclic complexes (fig. 5). This is demonstrated, as described above, by the position of the $\nu_f$(NH) band of acetamide at 3498 $cm^{-1}$ (fig. 6). However, the infrared spectra of equimolar solutions of these compounds do not allow us to choose among the possible structures in fig. 5. This is because the stretching vibrations of both carbonyl groups in uracil and the $\delta$NH vibration of this base are coupled [5,16]. Therefore, hydrogen bonding on one carbonyl and NH uracil groups may affect the frequencies and intensities of the other $\nu$C=O uracil band, and from the spectral changes in the 1750–1600 $cm^{-1}$ re-



(III) (IV)

Fig. 5. Structures of dimers between U and acetamide.

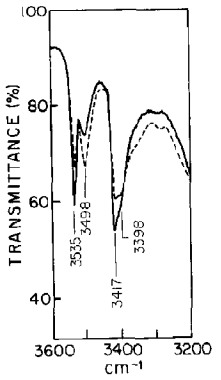



Fig. 6. (- - - - -) Infrared spectrum of an equimolar mixture of U and acetamide (0.12 M each). (———) Sum of the spectra of separated components. (0.1 mM KBr cell).

gion it is not safe to indicate which is the predominant heterodimer structure. However, $^{13}$C-NMR chemical shifts show conclusively that the heterodimers of type III structure are favoured in chloroform solution. Addition of acetamide to U solutions resulted in downfield shifts of the $^{13}$C-NMR resonance of the uracil C(4)=O group, showing that this group acts here as a hydrogen-bond acceptor (fig. 7). Simultaneous but smaller downfield shifts in the C(2)=O resonance imply

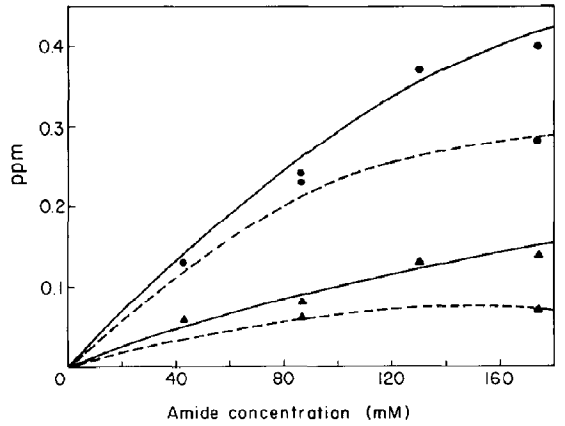



Fig. 7. (———) Downfield shifts of the $^{13}$C-NMR resonances of the U carbonyl groups on addition of acetamide. (●) C(4)=O group, (▲) C(2)=O group. The dashed lines correspond to the downfield shift of the $^{13}$C-NMR resonances of U carbonyl groups on addition of t-Boc-Gly-Gly-Gly-OBz, according to ref. 16.

that this carbonyl group of the uracil ring is a weaker hydrogen-bond acceptor than the C(4)=O group. On the other hand, comparison of these $^{13}$C-NMR shifts with those determined from mixed solutions of U and some tripeptides (fig. 7) [19] allows us to conclude that acetamide is more effective than peptide groups in forming hydrogen bonds with uracil bases.

In conclusion, the present spectroscopic data suggest a complementarity between adenine nucleobase and the *cis* part of the protein side-chain amide group, according to the formulation of fig. 1. In addition, infrared spectroscopic measurements of equilibrium constants have provided the first demonstration of a preferential order of associations: $k_1 > k_7$. Similarly, uracil base and the *cis* amide grouping form cyclic heterodimers in chloroform solution, the uracil C(4)=O carbonyl group being the most effective hydrogen-bond acceptor. The difference in hydrogen-bonding strength between N1 and N7 purine sites and between both uracil carbonyl groups gives rise to perturbation of the Watson-Crick nucleic base-pairs when amide species are present. Thus, the association constant between adenine and uracil [5] is about 5-fold greater than those of A-lactam cyclic heterodimers. This means that about 25% of base-pair hydrogen bonds in an equimolar (0.16 M) A-U solution are broken in the presence of lactam at the same concentration, as proved here. All of these results may explain the reduction in the melting temperature of the poly(A) · poly(U) helix [16] in the presence of acetamide, as the hydrogen-bond recognition of single-stranded poly(A) or poly(U) by the amide group is favoured. Although the single-stranded binding proteins need to stabilize exposed bases through the provision of aromatic protein side chains that participate in base stacking type interactions [20], our spectroscopic data suggest that hydrogen bonding between glutamine or asparagine side chains and adenine or uracil may contribute to such single-stranded stabilization to some extent. There are some reported crystal structures which serve as proof of this. An interaction between the glutamine amide group and adenine N1 and N(6)H binding sites has been described for complexes of pancreatic ribonuclease S and cytidylyl-2′,5′-

adenosine [21]. Brayer and McPherson [22] were able to cocrystallize ribonuclease with the oligonucleotide $d(pA)_4$, and the structure of the complex shows how the protein is able to immobilize the single-stranded nucleic acid and direct it to the active site [23,24]. Among other nucleobases close to the active center, adenine contributes to the binding of the protein through the above-mentioned binding sites. Apart from these cases of single-stranded nucleic acid-amide interactions, structural complementarity of type II (fig. 1) has been recently observed for A · T base-pairs interacting with glutamine in some repressor-operator complexes [25–28]. These complexes involve the DNA-binding domains of λ-repressor [25] and phage 434 repressor [26], as well as 434 Cro protein [27], and their crystal structures are beginning to yield structural information about sequence-specific contacts with the exposed parts of the operator base-pairs. In the λ complex [25], the $-NH_2$ group of glutamine residue $Gln^{44}$ donates a hydrogen bond to N7 of adenine and the amide accepts a hydrogen bond from the N6. Although this complex has a rather different DNA configuration and a different protein dimer interaction from that in the phage 434 repressor complex, it is remarkable that the character of many local interactions at homologous positions is conserved between the two structures. Thus, bidentate hydrogen bonding also links $Gln^{28}$ with analogous adenine (position 1) in the phage 434-repressor-operator complex [26]. Similar amide-purine contact occurs between $Gln^{28}$ and adenine in base-pair 1 of the crystalline complex of Cro protein and a synthetic operator [27]. However, the complex of the Trp repressor with operator [28] displays a different mode of interaction. In this case, there are no direct hydrogen bonds between Gln or Asn residues and adenine-thymine base-pairs that have been shown in vivo to be important in recognition. One reason for the absence of these cyclic hydrogen bonds is the fact that Gln or Asn residues in the complex are too distant from adenine bases for such contacts to occur. Comparing known cocrystal structures of repressor-operator complexes suggests that there is little prospect of any simple recognition code. It does not appear that there is any strict rule for determining how Gln or Asn will be used or how a particular base will be recognised. Repressor-operator complementarities include protein contacts with the sugar-phosphate backbone, as well as base-pairs, and the specificity seems to depend on all of these correlated interactions taken together [25–28]. However, recognition of adenine in A · T base pairs involves type II hydrogen bonds (fig. 1) [25–27] whenever these complexes possess the appropriate geometry for permitting a favourable approach and orientation of the Gln or Asn amide group with regard to those base-pairs.

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