FEBS Letters 432 (2001) 228–232 FEBS 24694

The C-terminus of dUTPase: observation on flexibility using NMR

Johan Nord[a,*], Per-Olof Nyman[a], Gunilla Larsson[a], Torbjörn Drakenberg[b]

[a] *Department of Biochemistry, Center for Chemistry and Chemical Engineering, Lund University, P.O. Box 124, S-22100 Lund, Sweden*
[b] *Physical Chemistry 2, Center for Chemistry and Chemical Engineering, Lund University, P.O. Box 124, S-22100 Lund, Sweden*

Received 27 November 2000; revised 5 February 2001; accepted 5 February 2001

First published online 2 March 2001

Edited by Thomas L. James

Abstract The dynamics of the C-terminus of the dUTPases from *Escherichia coli* and equine infectious anaemia virus (EIAV) were studied by ^{1}H–^{15}N nuclear magnetic resonance spectroscopy. The two enzymes differ with regard to flexibility in the backbone of the 15 most C-terminal amino acid residues, some of which are conserved and essential for enzymic activity. In the bacterial enzyme, the residues closest to the C-terminus are highly flexible and display a correlation time in the nanosecond time range. No similar high flexibility could be detected for the C-terminal part of EIAV dUTPase, indicating a different time range of flexibility.

Key words: dUTPase; Equine infectious anemia virus; Flexible C-terminus; Nuclear magnetic resonance; Nucleotide binding; *Escherichia coli*

1. Introduction

dUTPase is a ubiquitous enzyme catalysing the hydrolysis of the nucleotide dUTP to dUMP and inorganic pyrophosphate. The reaction is part of the biosynthetic pathway towards dTTP, and is crucial for maintaining the minimal ratio of dUTP to dTTP essential for the integrity of DNA. Being expressed in dividing cells [1] and indispensable for viability [2,3], dUTPase has been suggested as a target for drugs against cancer [4], as well as against viruses [5] that depend on a dUTPase of their own [6,7].

Structurally, the dUTPases can be classified into a few groups, the major one being represented by the homotrimeric dUTPases. These globular proteins are in the 50 kDa molecular mass range [8–10] and are similar with respect to enzymic properties, with high substrate specificity and low K_M for dUTP [11–13]. Several crystal structures have been obtained [14–17]. The discrimination against other pyrimidine nucleotides could be attributed to a hydrophobic pocket, the form and hydrogen bond characteristics of which being complementary to the deoxyuridine moiety of the substrate. In the majority of X-ray structures, a C-terminal portion of about 16 residues is not visible. This part of the protein, here referred to as the arm, is essential for enzymic activity [18–20]. It contains a conserved sequence reminiscent of a common motif with phosphate binding function [21]. A thorough characterisation of this C-terminal arm is a prerequisite for the assessment of the catalytic mechanism of dUTPase.

So far, the C-terminal arm has been characterised to a limited extent only. Measurements of the catalytic activity have been carried out on enzyme with the arm removed [18,20] or perturbed by alteration of individual amino acid residues [16,19]. The C-terminal arm of equine infectious anaemia virus (EIAV) dUTPase was shown to contribute significantly to neither the binding strength nor the association rate constant of the substrate, as monitored by stopped-flow fluorescence spectroscopy [20]. Measurements of properties of the C-terminal arm itself are, however, limited to an investigation by circular dichroism (CD) [22] and to a crystallographic study of an N-terminally truncated human dUTPase from which the co-ordinates have not been deposited [16]. The former study revealed a shift in CD upon binding of nucleotide to the *Escherichia coli* dUTPase, which was ascribed to the arm.

To characterise the C-terminus further, we have utilised nuclear magnetic resonance (NMR) to monitor the dynamics of the arm. The investigation comprises the dUTPases from *E. coli* and EIAV.

2. Materials and methods

2.1. Chemicals and enzyme preparations

Chemicals were of pro analysi quality (Merck, Sigma and Pharmacia). dUTP was purified, and 2′-deoxyuridine-5′-(α,β-imido)triphosphate (dUPNPP) [23] was checked for purity, by use of ion exchange chromatography [12]. Unlabelled recombinant *E. coli* and EIAV dUTPases were produced and purified as before [8,24]. *E. coli* dUTPase preparations were dialysed extensively against 81 mM sodium phosphate, 0.006% NaN_3, pH 6.0, and concentrated by use of centrifugal concentrators (Macrosep and Microsep, Pall Filtron) to a concentration of above 1 mM active sites. To test that the protein preparations would be stable during the NMR measurements, small samples were stored at 37°C for 36 h, after which their enzymic activity was assayed. Enzyme labelled with ^{15}N was prepared as above, except that the expression strain was grown at 25°C in M9 minimal medium [25], containing also $^{15}NH_4Cl$ (Martek, MD, USA), 0.1 mM $CaCl_2$ and 20 μM $FeCl_3$, and harvested 8 h post induction. The EIAV enzyme was treated identically except that dialysis was done with 60 mM sodium phosphate, pH 6.7, and 0.01% detergent ($C_{12}E_8$, monodisperse, Nikko Chemicals, Japan), or 8 mM sodium phosphate and 2 mM $MgCl_2$, pH 6.7, without detergent. This latter condition for the EIAV dUTPase preparation was selected by activity assay as the best out of several conditions with different magnesium chloride and sodium phosphate concentrations. No antibacterial agent was added to the preparations of EIAV dUTPase. Instead, the protein solutions were sterile filtered, and enzymic activity was assayed after the recording of NMR spectra.

2.2. Assays of protein and enzyme activity

Protein concentration was determined spectroscopically at 280 nm

*Corresponding author. Present address: AstraZeneca R&D Södertälje, Forskargatan 20 (B218), S-151 85 Södertälje, Sweden. Fax: (46)-8-553 28890. E-mail: johan.nord@astrazeneca.com

0014-5793/01/$20.00
PII: S0014-5793(01)02257-8

using molar extinction coefficients for EIAV and *E. coli* dUTPase of 15 300 [8] and 8500 M^{-1} cm^{-1}, respectively (for the monomers, i.e. active sites). In order to check for full activity of the enzyme preparations as well as to confirm the determinations of enzyme concentration, the rate of enzymic hydrolysis of 60 μM dUTP was monitored spectrophotometrically by following the pH dependent shift of cresol red in a weakly buffered solution of pH 8 [12]. The concentration of enzyme active sites was estimated from k_{cat} [12,13] and V_{max}.

2.3. NMR spectroscopy

The NMR samples contained protein, 0.8–1.3 mM active sites, in H_2O/D_2O (90/10) and a phosphate buffer (refer to Section 2.1 for concentrations). Solutions of 280 μl up to 320 μl were used in 5 mm Shigemi NMR tubes. The sensitivity enhanced gradient selected ^1H–^{15}N heteronuclear singular quantum coherence spectroscopy (HSQC) and ^1H–^{15}N total correlation spectroscopy (TOCSY)–HSQC [26] NMR spectra were recorded at 37°C on a Varian Unity Inova 600 with a proton frequency of 599.96 MHz, and a TOCSY spectrum on a GE Omega 500 spectrometer working at a proton resonance frequency of 500.10 MHz. The titrations with either dUDP (74 mM) or dUPNPP were performed, after addition of $MgCl_2$ to a concentration of 5 mM, by adding μl amounts of the solutions directly into the NMR tube by means of a thin plastic tube fitted to the tip of a Finnpipette. In this way, not removing the sample from the NMR tube, it was possible to perform the titration without any loss of sample.

3. Results and discussion

In most of the crystal structures for dUTPases reported [9,14,15,17], a C-terminal part of the polypeptide appears as unordered. It has frequently been suggested to be flexible [18,19,22,27], although the only experimental indication of flexibility is an observation of specific susceptibility towards tryptic digestion [22]. None, however, of the methods used so far to study the arm measures flexibility or rates of movement. Arguing that the C-terminal arm, as essential for catalytic activity, deserves more investigation, we utilised the NMR technique to study the rate of movement of the arm. Because the size of dUTPase gives rise to significant line broadening in NMR, signals from flexible parts of the protein should be distinguishable from signals originating from its rigid body.

Fig. 1A,B show a ^1H–^{15}N HSQC NMR spectrum of *E. coli* dUTPase. It is obvious that most of the resonances of this spectrum are broad as expected for a molecule with a molecular mass of 49 kDa. It is, however, also clear that there are, in addition to some sharp signals from side chain amide groups, a few resonances that are significantly sharper than others (Fig. 1B). Even though we have not made sequence specific assignments, it is clear from a HSQC–TOCSY spectrum (Fig. 1C) that the sharp resonances correspond to amino acid residues closely matching those of the C-terminal arm of the protein (see the legend to Fig. 1C). This finding shows that 15 amino acid residues of the C-terminus are flexible. The correlation time of the residues at, or close to, the C-terminal end is estimated to be less than 1 ns. The flexibility of the 15 C-terminal residues observed here agrees with the finding that these residues do not give rise to any interpretable electron density in crystallography [9,14,15,17]. In an attempt to assess the sequence of the amino acid residues giving rise to the strong peaks, a nuclear Overhauser enhancement spectrum with a mixing time of 200 ms was recorded. It did not, however, provide any useful information. This is mainly due to the fact that the broad peaks contribute strongly to the spectrum.

Addition of Mg^{2+}, cofactor in the catalysed reaction, has very little effect on the NMR spectrum. In fact, nothing in our

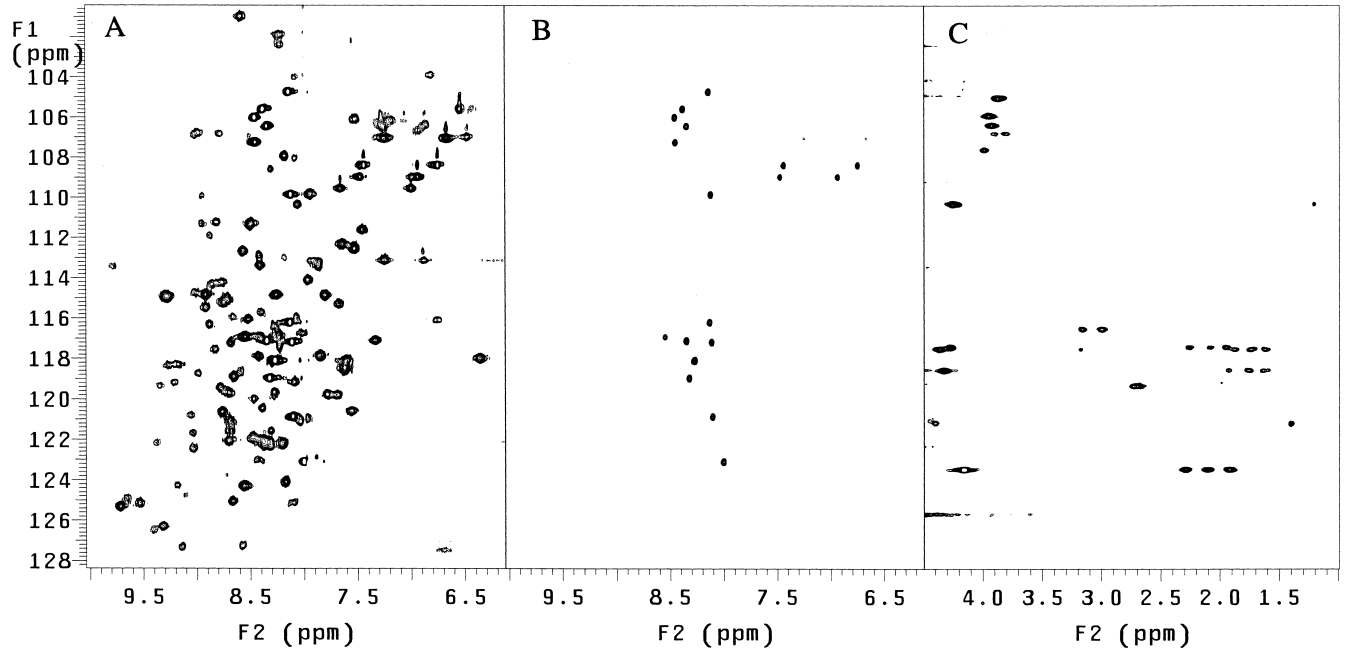

Fig. 1. A ^1H–^{15}N HSQC spectrum of *E. coli* dUTPase (1 mM active sites). An important point of (A) and (B) is to show that some of the resonances of the spectrum are clearly stronger than the majority of the peaks. The former have amplitudes well above the highest amplitude contour of the spectrum as shown here (they appear with a white interior). The difference in amplitude is even clearer in (B), in which the same spectrum is presented with a cut-off level 10-fold higher than that in (A), allowing only these strong peaks of the spectrum to be viewed. C: The side chain part of a HSQC–TOCSY spectrum of the sample in (A) and (B). The 13 strong backbone cross peaks are the only ones resulting in observable TOCSY cross peaks in the side chain region of the spectrum and can be assigned to, from top to bottom, 5 Glys, 1 Thr, 1 aromatic (Phe or His), 1 Glx, 2 Args, 1 Asp, 1 Ala and 1 Glx. These strong peaks correspond to the side chains of all, except two (Phe or His, and Ser), of the last 15 residues of the C-terminus (-ATDRG EGGFG HSGRQ) of *E. coli* dUTPase.

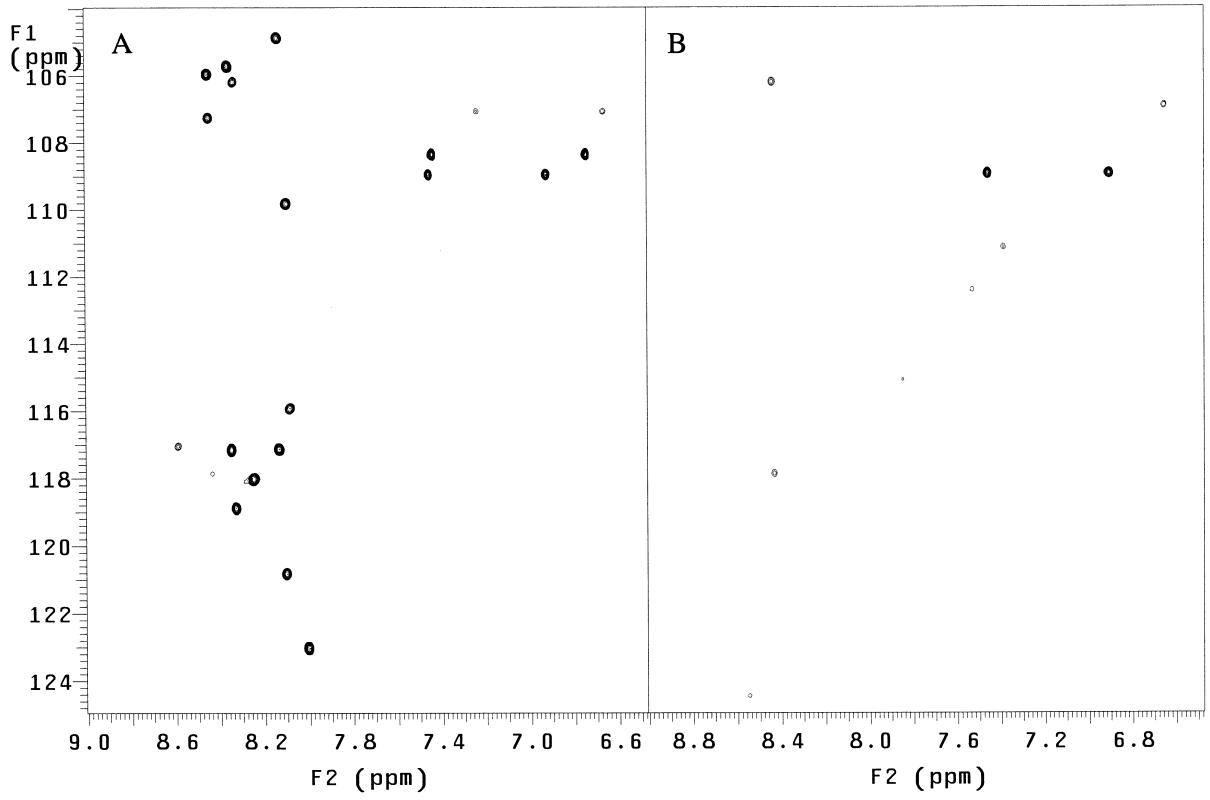

Fig. 2. ^{1}H–^{15}N HSQC spectra of *E. coli* dUTPase (1 mM active sites) with (A) the nucleotide dUDP added to a concentration of 4.4 mM, and (B) the nucleotide dUPNPP present at a concentration of ~1 mM. The cut-off level is the same as in Fig. 1B, and thus set to show only peaks of high amplitude. Note that the differences between the spectrum in (A) of this figure and the one in Fig. 1B (no nucleotide added) are very small, while in (B) of this figure the amplitudes of the resonances have decreased dramatically.

data suggests that a metal ion is bound to the C-terminal arm. In a subsequent titration with dUDP, it was observed that even though the resonances from the arm stayed essentially unaffected (Fig. 2A), significant changes in the chemical shifts of some of the broader resonances were observed (data not shown). These changes resulted in reasonable titration curves (data not shown), corresponding to the presence of *one* binding site for dUDP in each *E. coli* dUTPase monomer, which is in agreement with earlier observations (e.g. [12]). More important, however, is the observation that the arm in the *E. coli* dUTPase is as flexible with a bound dUDP as without.

The active site inhibitor dUPNPP binds strongly to dUTPase ($K_d = 5 \times 10^{-6}$ M [12]). With the high concentration of enzyme needed for NMR, the binding affinity cannot be determined. The effect on the spectrum caused by the inhibitor is, however, dramatic as can be seen from Fig. 2B. All the sharp resonances from the arm are broadened upon inhibitor binding and, furthermore, the broadening is substantial already after the addition of much less than one equivalent of inhibitor, showing that the exchange rate of the inhibitor is in the intermediate range on the NMR time scale ($k_{off} = 10^3$ s^{-1}). In the presence of excess of inhibitor, almost all resonances from the arm are as broad as the other resonances, showing that the arm is now essentially fixed (the correlation time of the residues in the arm has increased to around 10^{-7} s). This shows that the third phosphate group (the γ-phosphate) in the nucleoside triphosphate is essential for binding the arm as it is for enzymic catalysis. We therefore suggest that the reason that dUDP is not a substrate, even though the bond corresponding to the cleavage site in dUTP is present, is due to its inability to fix the arm. These observations are similar to what was found previously by the use of CD [22]. In that study, a shift in far-UV CD appeared only with dUPNPP, not with dUDP, bound to the enzyme. The shift was dependent upon the presence of Mg ions, but more importantly, it failed to appear when the nucleoside triphosphate was mixed with modified *E. coli* dUTPase, the C-terminal arm of which had been removed. These findings support our conclusion that the stronger peaks in the NMR spectra originate from amino acid residues in the arm.

The present investigation shows that association with the enzyme of nucleotides, also those that do not interact with the C-terminal arm, can be followed using the NMR method. When a detailed study of dUTPase by NMR has been conducted, with a full assignment of the resonances of the protein spectrum, chemical shifts induced by a nucleotide such as dUDP (see above) would be possible to interpret in terms of interactions of the nucleotide with specific amino acid residues in the active site of the enzyme.

In general, dUTPases of various origins show similarities in catalytic properties, a finding taken as an indication for a common catalytic mechanism [27]. The two dUTPases most thoroughly characterised in this regard, the ones from *E. coli* and EIAV, differ, however, in one respect. For the EIAV dUTPase, the binding of dUPNPP is not significantly affected by removal of the C-terminal arm [20], while in the *E. coli*

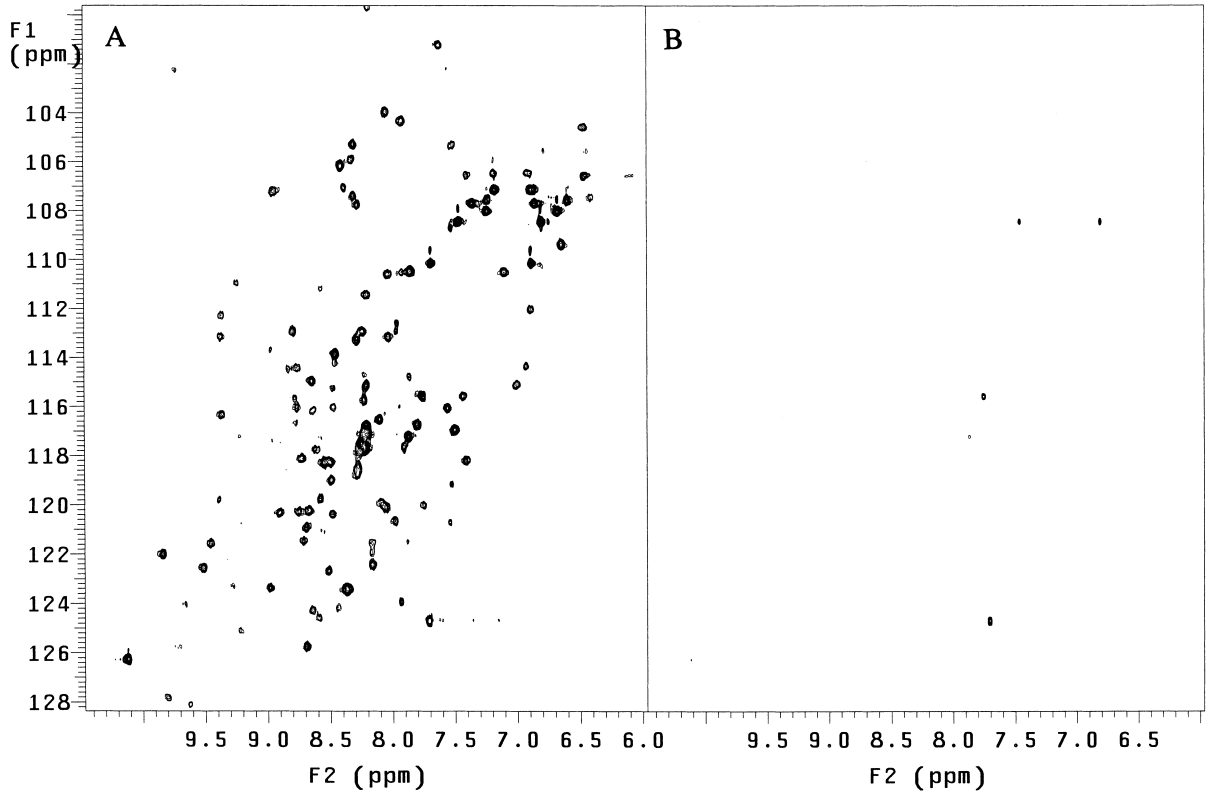

Fig. 3. A ^{1}H–^{15}N HSQC spectrum of EIAV dUTPase (1 mM active sites) presented, as for *E. coli* dUTPase in Fig. 1A,B, with (A) a low cut-off level that shows all the peaks of the spectrum and (B) with a cut-off level 10-fold higher (as in Fig. 1B) allowing only resonances of high amplitude to be displayed. Note the lack of flexibility in this enzyme in contrast to the flexibility indicated by the corresponding spectrum of the *E. coli* dUTPase (Fig. 1B).

enzyme, the binding strength becomes somewhat decreased (by about one order of magnitude) [22]. These results were obtained using different experimental methods and the possibility of artefacts cannot be excluded. A comparison of the two enzymes in NMR properties was considered desirable.

A HSQC spectrum was acquired for the EIAV dUTPase without nucleotide added (Fig. 3). There is a striking difference between this spectrum and the corresponding one for the *E. coli* dUTPase shown in Fig. 1A,B. In the spectrum of EIAV dUTPase, sharp signals were observed only for two main chain amide groups. Therefore, it is clear that the arm behaves differently in the two proteins, even though X-ray crystallography indicates that the arm is unordered in both proteins. Kinetic data of nucleotide binding obtained earlier by stopped-flow measurements indicate that the movement of the arm in the EIAV enzyme is fast compared with the diffusion rate limited association of the nucleotide ligand [20]. Together with the NMR data, this would suggest that the rate of movement of the arm in the EIAV dUTPase is in the μs time range, in contrast to the ns range observed for the C-terminal residues in the bacterial enzyme. The slower speed of the arm in the EIAV enzyme cannot per se be taken as an indication of a reduction in the degrees of freedom relative to the situation in the bacterial enzyme. The difference between the two enzymes may be trivial and primarily a question of rate of movement of the arm. However, the similarity between dUTPases in the behaviour and role of the arm assumed previously might need corroboration by further experiments.

A possible reason for the slower rate of movement of the arm in the EIAV enzyme would be interactions of the arm with the globular part of the enzyme, a situation that would conceivably reduce the translational and rotational entropy of the arm relative to the arm in the *E. coli* dUTPase. Another possibility with a similar entropic outcome would be an internal structure of the arm present during movement. Such possibilities should be considered in the light of the differences between the two enzymes in C-terminal amino acid sequence.

The present investigation demonstrates NMR as a tool for study of dUTPase. This is particularly valid for dUTPases with a highly flexible C-terminal arm, e.g. *E. coli* dUTPase. The flexibility suggested by previous investigators has been given a more precise and quantitative nature. The methods for over-expression and purification recently adapted for dUTPases in our laboratory [8,24] have proven to be suitable for preparation of isotope labelled proteins. The results suggest that future use of the NMR technique for investigations of dUTPase should be rewarding.

Acknowledgements: We would like to express our gratitude to the late Professor A. Krayevsky, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, for providing the substrate analogue dUPNPP, and to Dr. T. Persson, Växjö University, Växjö, Sweden, for assistance in its synthesis. This work was supported by the European Commission BIOMED-2 program (contract number BMH 4CT97-2711 (DG12-SSMI)), by the Swedish Cancer Society, and by the Swedish Medical Research Council. During the preparation of the present manuscript, a crystallographic study of the feline immunodeficiency virus dUTPase, including its C-terminal arm, has been published, the results of which suggest an important role for the C-terminal arm in the catalytic mechanism and emphasise the need for further studies of this part of the enzyme [28].

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