Thermodynamic characterization of the binding of dCMP to the Asn229Asp mutant of thymidylate synthase

Ramiro Téllez-Sanz[a], Víctor Bernier-Villamor[b], Luis García-Fuentes[a,c], Dolores González-Pacanowska[b], Carmen Barón[a,c,*]

[a]*Departamento de Química Física, Bioquímica y Química Inorgánica, Facultad de Ciencias Experimentales, Universidad de Almería, La Cañada de San Urbano, 04120 Almería, Spain*
[b]*Instituto de Parasitología y Biomedicina, 'López Neyra', C.S.I.C., 18071 Granada, Spain*
[c]*Instituto de Biotecnología de Granada, Granada, Spain*

Received 11 March 1997; revised version received 25 April 1997

Abstract Isothermal titration microcalorimetry and equilibrium dialysis have been used to characterize the binding of 2′-deoxycytidine 5′-monophosphate (dCMP) to the Asn229Asp mutant of *Lactobacillus casei* recombinant thymidylate synthase at pH 7.4 over a temperature range of 15°C to 35°C. Equilibrium dialysis analysis shows that dCMP binds to two sites in the dimer of both wild-type and mutant thymidylate synthase. A concomitant net uptake of protons with binding of dCMP to both enzymes, was detected carrying out calorimetric experiments in various buffer systems with different heats of ionization. The change in protonation for binding of dCMP to wild-type enzyme is lower than that obtained for binding of this nucleotide to TS N229D, which suggests that the pK value of Asp-229 is increased upon dCMP binding to the mutant enzyme. At 25°C, although the binding of dCMP to wild-type and N229D TS is favoured by both enthalpy and entropy changes, the enthalpy change is more negative for the mutant protein. Thus, the substitution of Asn 229 for Asp results in a higher affinity of TS for dCMP due to a more favourable enthalpic contribution. The Gibbs energy change of binding of dCMP to the mutant enzyme is weakly temperature-dependent, because of the enthalpy-entropy compensation arising from a negative heat capacity change of binding equal to -0.83 ± 0.02 kJ K^{-1} per mol of dCMP bound.

Key words: Asn229Asp mutant of thymidylate synthase; dCMP; Microcalorimetry; Equilibrium dialysis; Binding

1. Introduction

Thymidylate synthase (TS; EC 2.1.1.45) catalyzes the reductive methylation of deoxyuridine 5′-monophosphate (dUMP) to produce deoxythymidine 5′-monophosphate (dTMP), with the cofactor 5,1-methylenetetrahydrofolate (CH_2H_4folate) being converted to dihydrofolate (H_2folate). TS provides the only de novo pathway for dTMP production and has received much attention as a drug target. Molecular details of the catalytic mechanism and structure of TS are now well understood [1]. In *Lactobacillus casei*, the enzyme is a dimer of identical subunits of 36 kDa, each of which contains an active site. The three-dimensional structure of TS bound to DUMP has been solved at 2.55 Å resolution [2]. In this binary complex, Asn 229 is involved in a cyclic hydrogen bond network with the pyrimidine ring of dUMP [2–4]. The mutation of amino acids of the active site have allowed to elucidate the role of specific residues in binding and catalysis [5,6]. The analysis of several mutants at position 229 of *L. casei* TS [7,8] showed that N229 assists but is not essential for dUMP binding or for catalysis. Wild-type TS binds dCMP weakly and does not catalyze the methylation of dCMP. However, the Asn229Asp mutant of *Lactobacillus casei* thymidylate synthase (TS N229D), as well as the corresponding Asn177Asp mutant of *Escherichia coli* TS, present altered substrate specificity and catalyze preferentially the methylation of dCMP versus dUMP [6–8]. The crystal structures of wild-type TS and TS N229D bound to dCMP have been recently solved [9] and have revealed the structural basis for the change in specifity.

In order to understand the forces driving association between dCMP and TS N229D, we have studied ligand binding using isothermal titration calorimetry (ITC) and equilibrium dialysis. The calorimetric experiments were performed at several temperatures and showed that ΔH_b is negative at 35°C and decreases linearly over the temperature range from 15°C to 35°C, given a negative value of ΔC_p. ITC was also employed to study the thermodynamics of interaction between dCMP and wild-type TS at 25°C. The heat measured was found to depend on buffer ionization heats in both cases, reflecting that complex formation is accompanied by uptake of protons. During binding, protonation of the TS N229D-dCMP complex is higher than that of the TS-dCMP complex, which suggests that Asp-229 may be involved in the proton uptake. The results are discussed in the light of the structural knowledge of the complexes of dCMP with the wild-type and N229D enzymes.

2. Materials and methods

Thymidylate synthase was purified from the Thy$^-$ *E. coli* strain χ_{2913} transformed with pKPTSd, which contains the complete coding sequence for the TS of *Lactobacillus casei* [10]. Purification involved sequential chromatography on phosphocellulose and hydroxylapatite as described by Kealey and Santi [11].

Asn229Asp mutant of thymidylate synthase (TS N229D) was purified from χ_{2913}/pSCTS9 N229D. After purification, wild-type and mutant were concentrated to 15–20 mg/ml and the buffer changed to 10 mM KH_2PO_4 (pH 6.8) 0.1 mM EDTA. Both enzymes showed a single-band pattern in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and dCMP deaminase activity has not

*Correspondence author. Fax: (34) (50) 215008.
E-mail: cfbaron@ualm.es

Abbreviations: H_2folate, dihydrofolate; dCMP, 2′-deoxycytidine 5′-monophosphate; dUMP, 2′-deoxyuridine 5′-monophosphate; ITC, isothermal titration calorimetry; CH_2H_4folate, 5,10-methylenetetrahydrofolate; Pipes, 1,4-piperazinediethanesulfonic acid; dTMP, thymidine 5′-monophosphate; TS, thymidylate synthase; TS N229D, Asn229Asp mutant of thymidylate synthase

PII S0014-5793(97)00551-6

been detected. Purified proteins were stored at −80°C. The purification yield was around 40 mg and 35 mg of apparently pure protein per l of culture for wild-type and mutant TS, respectively.

Solutions of wild-type and N229D mutant were prepared by dialysis of the enzyme against several changes of 50 mM KCl, 1 mM 2-mercaptoethanol, 1 mM EDTA, 50 mM of buffer solution (Pipes, HEPES or Tris) at pH 7.4 and 4°C. Protein concentrations were determined from absorbance measurements at 278 nm, using the absorbance coefficients of 1.256×10^5 M^{-1} cm^{-1} [12]. TS [13] and dCMP methylase [7] activities were measured spectrophotometrically by monitoring the increase in absorbance at 340 nm, which accompanies H_2folate production. Reaction mixtures contained 0.1 mM CH_2H_4folate, 0.125 mM dUMP and 10–14 nM protein for TS or 0.340 mM CH_2H_4folate, 0.4 mM dCMP and 10–14 nM protein, for dCMP methylase. One unit of activity is defined as the amount of enzyme necessary to synthesize 1 μmol of product in 1 min at 25°C for TS and 20°C for TS N229D. Specific activities of purified wild-type and N229D TS were 2.5–3 units/mg and 0.2–0.3 units/mg, respectively. Absorbance measurements were carried out in a Beckman DU-7400 spectrophotometer with the cells maintained at 25°C.

All chemicals used were of the highest purity available and purchased from either Merck or Sigma. [2-^{14}C]dCMP was obtained from Moravek Biochemicals, Inc. Centriprep 30 concentrators were from Amicon.

2.1. Techniques

The equilibrium dialysis experiments were performed at 25°C using a Dianorm equilibrium dialysis system with Spectrapor 12–14 kDa molecular mass cut-off membranes as described in elsewhere [14]. A LKB wallac 1209 Rackbeta scintillation counter was used in the dialysis experiments to calculate the saturation fraction of the enzymes with dCMP.

ITC experiments were performed in a calorimeter built in our laboratory, interfaced to a microcomputer using an A/D converter board (Data Translation DT-2805) for automatic instrument control and data collection. The characteristics of this instrument are similar to other calorimeters, such as those described by McKinnon et al. [15] and Freire et al. [16]. The titration experiments were carried out at pH 7.4 and 15°C, 25°C and 35°C, as described elsewhere [17,18]. All appropriate corrections for heats of dilution were applied. The thermal effect of the protein dilution was negligible in all cases. The activity of the enzyme was routinely checked just before and after the calorimetric experiment. Similarly, the pH values of the buffer, dCMP, and protein solutions were controlled at each temperature before and after the binding reaction.

It was assumed that the dimeric protein has two equal and independent sites for binding the ligand, L, with its characteristic microscopic association constant, K, and molar enthalpy change of binding ΔH_b, and with n_{H^+} being the number of protons taken up ($n_{H^+} > 0$) by the protein-ligand complex during the binding reaction. The heat released or absorbed for each ligand concentration, is given by the equation [18]:

$$Q = 2[P]\ V(\Delta H_b + n_{H^+}\ \Delta H_i)\frac{K[L]}{1+K[L]} \quad (1)$$

where [P] stands for the dimer protein concentration in the calorimeter cell, V for the reaction volume, and ΔH_i the heat of buffer ionization. The free ligand concentration after each injection must be calculated from these values according to:

$$[L] = \left\{\sqrt{(1-K[L]_T+2K[P])^2+4K[L]_T} + K[L]_T - 2K[P] - 1\right\}/2K \quad (2)$$

where $[L]_T$ is the total ligand concentration.

Eq. 1 involves three fitting parameters: ΔH_b, K and n_{H^+}. Once the convergence criteria is satisfied the values of those parameters can be obtained for each temperature. A computer program for specific data analysis has been written including a function optimization based on the Levenberg-Marquardt algorithm [19]. Heat capacity change of binding was obtained by measuring the enthalpy change on association over a range of temperature from 15°C to 35°C.

3. Results and discussion

3.1. Equilibrium dialysis measurements

The binding of dCMP to TS N229D was observed as a function of the nucleotide concentration by equilibrium dialysis at pH 7.4 and 25°C. The dimeric TS N229D concentrations were from 0.164 mM to 0.465 mM. The results of this binding study are displayed in Fig. 1 as a plot of degree of binding, ν, versus log[dCMP]. Extrapolation of higher part of the plot leads to $\nu = 2$ for TS N229D. A Scatchard plot the data clearly extrapolates to $\nu = 2$ (data not shown). No systematic deviations from a linear function are detected. These results may be taken as an evidence for the existence of two equivalent and non-interacting sites in the TS mutant. The fitting of the dialysis data to this model gave the optimum value $K = (10 \pm 0.5)\ 10^3\ M^{-1}$ and the theoretical ν data predicted with this value are drawn with the *solid line* curve in Fig. 1. An analogous study performed with dimeric wild-type enzyme have also showed two sites for binding of dCMP.

3.2. Isothermal calorimetry experiments

Direct calorimetric measurements were performed in order to obtain independent estimates of the thermodynamic parameters governing binding of dCMP to TS N229D and wild-type enzyme. Fig. 2A shows a typical ITC profile for the binding of dCMP to dimeric TS N229D in buffer Tris at pH 7.4 and 25°C. The sample data represent twelve equivalent 30 μl injections (spaced at 4 min intervals) of dCMP solution into the enzyme solution. A thermogram for the binding to wild-type TS at the same pH and temperature is shown in Fig. 3A. The positive sign of the measured heat indicates that the enthalpy change for each injection was positive and that the process of binding under these conditions was endothermic. Control experiments involved the same number of 30 μl injections of

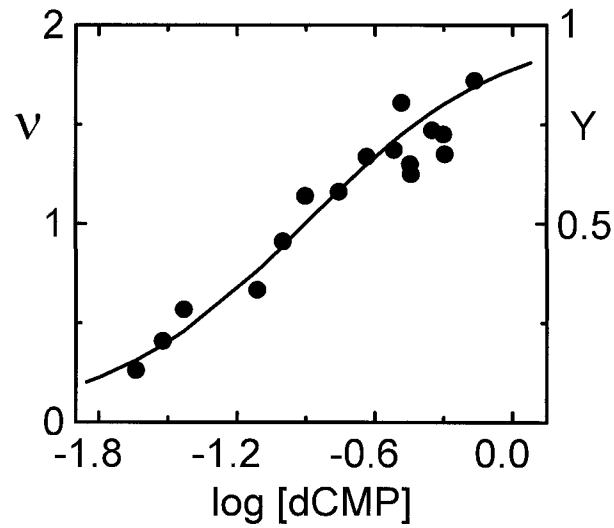

Fig. 1. Binding of dCMP to the Asn229Asp mutant of *L. casei* recombinant thymidylate synthase at pH 7.4 and 25°C. The data were obtained by equilibrium dialysis. The saturation fraction, Y, and the moles of dCMP bound per mol of protein, ν, are plotted vs. log[dCMP]. The enzyme concentration was between 0.164 mM and 0.465 mM TS N229D and the buffer solution used was 50 mM HEPES, 50 mM KCl, 1 mM EDTA and 1 mM 2-mercaptoethanol. The solid line gives the best fit to the data for a binding model of dCMP to two equivalent and non-interacting sites, with $K = (10 \pm 0.5) \times 10^3\ M^{-1}$.

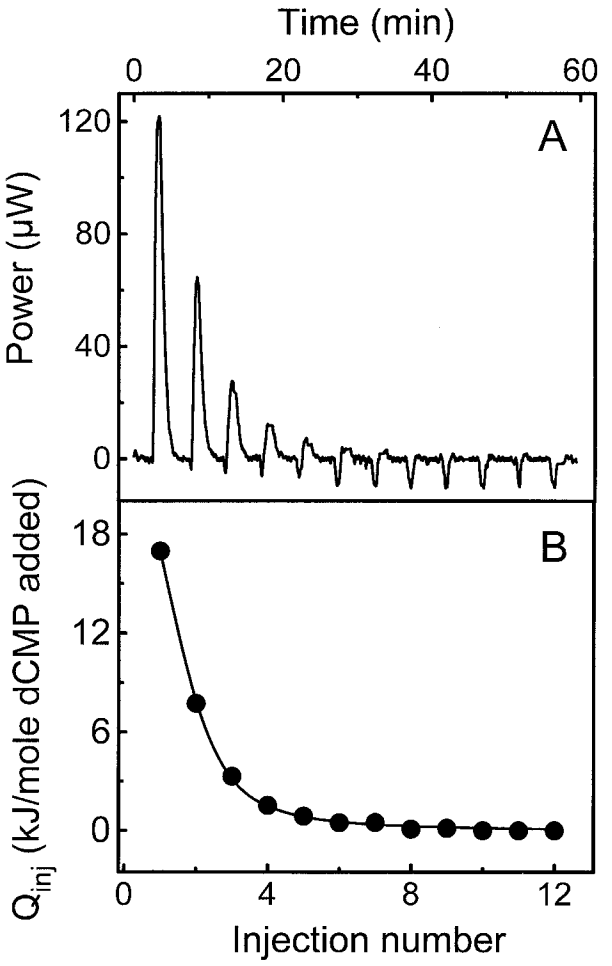

Fig. 2. Calorimetry titration of the binding of dCMP to the Asn229-Asp mutant of *L. casei* recombinant thymidylate synthase at pH 7.4 and 25°C in Tris buffer. (A) The programmed sequence consisted of 12 injections of 30 μl each of 21.72 mM dCMP stock solution. The dCMP and buffer solution were injected into a sample cell containing 2.9 ml of 137 μM dimeric TS N229D and reference cell containing 2.9 ml of buffer. The data shown in (A) are the differences between the sample cell and the reference cell. (B) Integrated heat change for each injection per mole of dCMP injected after subtracting the control injection is plotted vs. injection number. The solid line is the theoretical one for the parameter values shown in Table 1 and for 0.65 protons.

nucleotide solution into the same buffer although without enzyme present. Control injections represent the dilution heat of dCMP and the heat effects from non-chemical reaction sources, which have not been compensated with dual injection into the sample and reference cells. The negative deflections observed at the end of the titration in Fig. 2A are very similar to the control injections and show that saturation has been reached, while the last injections in the Fig. 3A are slightly different to the control injections and show that saturation has not been attained. The area under each peak is the heat for each injection. The integrated heats after subtraction of the small heat of the control experiment are divided by the moles of dCMP injected, and the resulting values are shown vs. injection number in Figs. 2B and 3B. Identical experiments to those described above were carried out in other buffers (HEPES and Pipes) at 25°C. The enthalpy changes of the binding strongly depended on the ionization heat of the buffer, which demonstrates linkages between dCMP and proton binding. Since the reaction is more endothermal in Tris buffer than in Pipes buffer for the two systems studied, we conclude that there is a net proton uptake during the binding reaction of the nucleotide to the wild-type and mutant enzymes.

The equilibrium dialysis data referred to above show that two mol of dCMP bind to wild-type and mutant TS in the range of ligand concentration studied.

There are uncertainties in literature with regard to the number of binding sites and in relation to their symmetry once the ligands have bound. So, for some ligands it has been shown the existence of two interacting binding sites [20,21], whilst other ligands seem to bind either to one binding site [22] or to two non-interacting binding sites [8,17]. From these findings it can be seen that the results obtained depend upon the system and the experimental studied conditions [23].

The analyses of all the experiments performed were consistent with a stoichiometry of two non-interacting binding sites in the wild-type and mutant proteins for the binding of nucleotide in agreement with the equilibrium dialysis studies. All titration data sets in different buffers were analyzed simultaneously assuming a value of 2 for the number of binding sites and using the ionization heat of the buffers [24]. The thermodynamic parameters obtained by non-linear least squares fitting of Eq. 1 to the experimental data are given in Table 1 for wild-type and mutant TS and the number of protons uptaken per subunit of wild-type TS and TS N229D during dCMP binding were 0.47 ± 0.03 and 0.65 ± 0.01, respectively, at 25°C. Gibbs energy and entropy changes for nucleotide binding were obtained from the microscopic binding constants and enthalpy changes at 25°C, and these functions are also displayed in Table 1. The standard state is that of 1 mol l^{-1}. The calculation of thermodynamic functions implies the usual approximation of setting standard enthalpies equal to the observed ones. The K value obtained by calorimetry for the mutant enzyme was equal within experimental error to that obtained by equilibrium dialysis at that temperature and under the same experimental conditions (Fig. 1). The constant values obtained for both proteins are lower than those given by Liu and Santi [8] and this discrepancy may be due to the difference in ionic strength, which suggests that electrostatic interactions are involved in the binding process [25]. At 25°C, although the binding of dCMP to both proteins was favoured both enthalpy and entropy-ly, the enthalpy change for the binding of the nucleotide to the mutant enzyme is more negative than that for the wild-type enzyme, while the ΔS^0 is

Table 1
Apparent thermodynamic parameters for the binding of dCMP to *L. casei* recombinant thymidylate synthase at pH 7.4 and 25°C

TS	$K \times 10^{-3}$ (M^{-1})	ΔG^0 (kJ mol^{-1})	ΔH_b (kJ mol^{-1})	ΔS^0 (J K^{-1} mol^{-1})
Wild type	3.0 ± 0.2	-19.8 ± 0.2	-2.43 ± 0.04	58 ± 1
N229D	13.5 ± 0.8	-23.6 ± 0.2	-4.30 ± 0.10	65 ± 1

The uncertainties are standard errors in fitting of the curves.

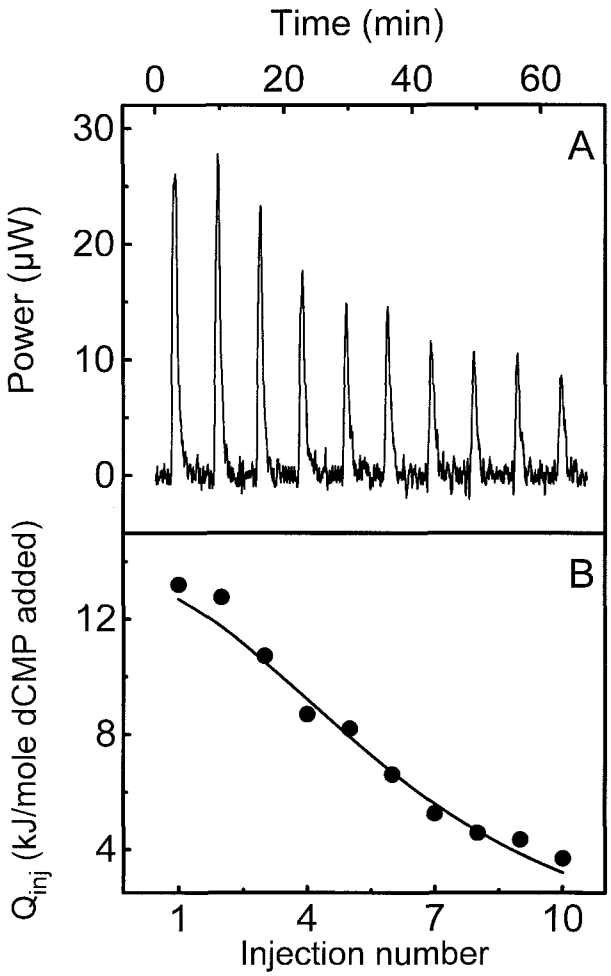

Fig. 3. Calorimetric titration of *L. casei* recombinant thymidylate synthase with dCMP at pH 7.4 and 25°C in Tris buffer. (A) shows the data for 10 injections of 30 μl each of a 3.15 mM dCMP solution into a sample cell containing 2.9 ml of 97 μM enzyme, while (B) shows integrated data per mole of dCMP injected after subtraction of control vs. injection number. The solid curve is the theoretical one for the parameter values shown in Table 1 and for 0.47 protons.

similar. This higher negative contribution to Gibbs energy change is responsible for the higher affinity displayed by N229D TS towards the nucleotide.

The ionization changes during nucleotide-enzyme binding can be attributed to a shift in the pK of one or more groups of the nucleotide and/or enzyme on the complex forms. Alterations in the protonation state of certain residues in the vicinity of the dCMP binding site may explain the uptake of protons when the nucleotide binds to the enzyme at pH 7.4. The formation of dCMP complex with TS N229D is accompanied by an uptake of approximately 0.2 H^+ *more* than that with wild-type TS. Since the only difference between both enzymes is the Asp-229 mutation, this result suggests that the pK of Asp-229 is raised upon binding, which correlates very well with our present knowledge of the structural information of the complex. Thus, Finer-Moore et al. [9] have proposed the formation of a hydrogen bond between the Asp-229 and 3-N on dCMP, which requires that Asp-229 is protonated. Stabilization of the imino tautomer by hydrogen bonding is necessary for to explain the mechanism of methylation of dCMP by TS N229D proposed previously [9]. The ΔH_b values (Table 1), although corrected for the enthalpy of buffer protonation, include the enthalpy change when 2′dCMP binds to the enzyme and the heat induced by proton uptake from the ligand-protein complexes. If Asp-229 uptakes approximately 0.2 H^+, its contribution to ΔH_b will be of the order of −1 kJ/mol at 25°C. The values of thermodynamic parameters could be analyzed in the light of the structural knowledge of dCMP complexes with wild-type and N229D TS [9]. The negative binding enthalpy values (Table 1) correlate well with the formation of hydrogen bonds postulated. As in the TS-dUMP complex, Arg-23, Arg-218, Arg-178′ and Arg-179′ as well as Ser-219 are involved in an extensive hydrogen-bonding network around the phosphate of dCMP [2,9,26]. Some of these highly conserved arginine residues, Arg-23 and Arg-218, have been shown to be important for activity [5]. Tyr-261 and His-259 are hydrogen bonded to the ribose ring. The possible formation of hydrogen bonds between Asp-229 and dCMP may explain the binding enthalpy difference between wild-type TS and TS N229D. A minor favourable enthalpy contribution is found for the binding of dCMP to wild-type TS with respect to the binding of dUMP [27]. This enthalpy difference may be attributable to the loss of hydrogen bonds in the dCMP complex.

3.3. Temperature dependence of the dCMP-TS N229D interaction

An analogous study to that described before was performed with TS N229D at 15°C and 35°C. The number of protons taken up by TS N229D subunit during dCMP binding were 0.620 ± 0.010; 0.681 ± 0.002 at pH 7.4 for 15°C and 35°C, respectively. Thus proton exchange varied only slightly with temperature. Consequently, at pH 7.4 and within the temperature range studied, the ΔH_b value practically does not change with pH.

The heat, Q, on the total amount of ligand added (Eq. 1) divided by the moles of dimeric protein, ΔH, was corrected for heat of ionization of buffer during the binding according to the Equation

$$\Delta H_c = \Delta H - 2n_{H^+}\,\Delta H_i \frac{K[\mathrm{dCMP}]}{1+K[\mathrm{dCMP}]} = 2\,\Delta H_b \frac{K[\mathrm{dCMP}]}{1+K[\mathrm{dCMP}]} \quad (3)$$

and three single sets of calorimetric values, ΔH_c, were obtained, one for each temperature. As the temperature rises the binding enthalpy becomes more exothermic. The solid lines in Fig. 4 are those calculated with Eq. 3, where the free concentration of dCMP is given by Eq. 2 for the thermodynamic parameters obtained, which are plotted as a function of temperature in Fig. 5. The enthalpy change upon binding decreases linearly with temperature in the range of 15°C to 35°C. Thus, a constant heat capacity change, ΔC_p, of -0.83 ± 0.02 kJ K^{-1} per mol of dCMP bound was obtained from the slope of a linear-regression analysis of the ΔH_b vs. T data (Fig. 5).

The enthalpy and entropy changes of TS N229D-dCMP complex formation depend strongly on temperature in the range of 15–35°C, while ΔG^0 changes little with temperature because of enthalpy-entropy compensation, as has been found for many ligand-protein interactions [18,28–30]. ΔH_b is negative at 25°C and 35°C, changing sign near 15°C and ΔS^0 is

positive within the 15–35°C temperature range. Thus, at 35°C, the binding of dCMP to TS N229D is driven almost equally by enthalpy and entropy as judged by their contributions to the ΔG^0 and by entropic components at 15°C. The positive entropy change can be explained by hydrophobic effects and electrostatic interactions. In the nucleotide binding reaction, the complex formation requires the dehydration of both the protein and the ligand and there is a entropic gain from transfer of interfacial water into the bulk solvent. Heat-capacity change negative values are usually interpreted as arising from the burying of apolar groups from water [31–36]. Murphy and Freire [37] and Spolar and Record [38] have suggested that the ΔC_p may be described as a phenomenon in hydration terms, pointing out that changes in vibrational modes apparently contribute little to ΔC_p. If there are changes in the ionization, their contribution to ΔC_p must be taken into account [39]. The binding of dCMP to TS N229D is accompanied by the uptake of approximately 0.65 protons within 15–35°C. The functional groups responsible for this proton uptake might be Asp-229 and, largely, other groups with low ionization heat. Thus, the contribution of protonation to ΔC_p is expected to be small. The application of Murphy's approach [37] to the experimentally determined values (Table 1) indicates that the surface area buried on complex formation comprises 60% non-polar surface (approximately 700 Å^2) and 40% polar surface (approximately 450 Å^2).

The results presented in this study show that complex formation of dCMP with both wild-type and N229D mutant TS is accompanied by a proton uptake and a comparison of both systems suggests that the pK value of Asp-229 is increased upon binding. On the other hand, our results indicate that the change of Asn-229 to Asp gives rise to an increased affinity of TS for dCMP due to a more favourable enthalpic contribution. The binding of dCMP to N229D TS is enthalpy and entropy driven at 35°C, while the binding is driven by entropic contributions at 15°C. Thus, the enthalpy and entropy

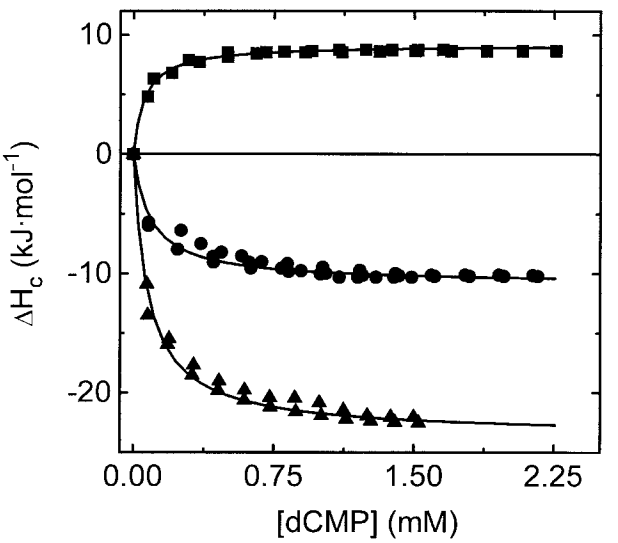

Fig. 4. Thermal titration of the Asn229Asp mutant of *L. casei* recombinant thymidylate synthase with dCMP at pH 7.4. Experimental values are corrected for the thermal effect of the protons taken up by the buffer systems. The corrected heat data are plotted as a function of the free dCMP concentration at 15°C (■), 25°C (●) and 35°C (▲). The solid lines are the theoretical ones corresponding to Eq. 3 and obtained from the values shown in Table 1.

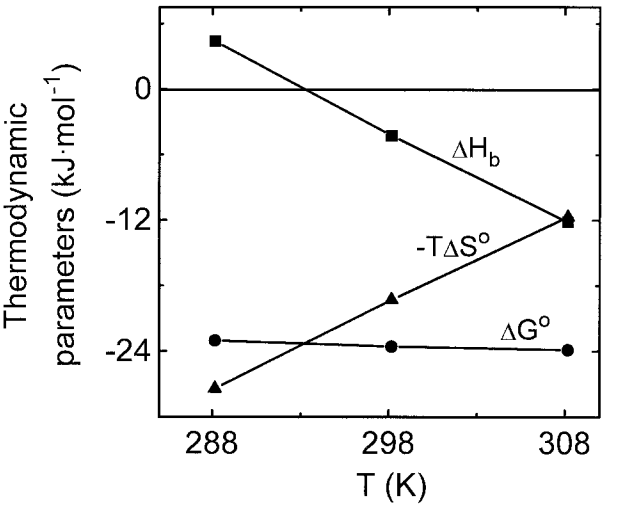

Fig. 5. Temperature dependence of the thermodynamic parameters for the binding of dCMP to the Asn229Asp mutant of *L. casei* recombinant thymidylate synthase at pH 7.4. The solid lines are the linear least-squares fitting of the data. The heat-capacity change, given by the slope of the plot of ΔH_b vs. T, is -0.83 ± 0.02 kJ K^{-1} per mol of dCMP bound.

changes upon binding exhibit strong temperature dependencies, arising from a significant negative heat-capacity change, whereas the affinity of the nucleotide is practically the same within the 15–35°C temperature range. The negative ΔC_p value suggests changes in buried hydrophobic and hydrophilic areas upon nucleotide binding, with liberation of water molecules from both the protein and the nucleotide.

Acknowledgements: We would like to thank Dr. O. López-Mayorga for the excellent technical assistance and the Biocalorimetry Center (NIH) of The Johns Hopkins University for their technical help with the construction of the titration calorimeter. The authors gratefully acknowledge Dr. D.V. Santi for providing plasmids pKPTSd and pSCTS9 which express the wild-type and N229D mutant, respectively.

This research was supported by Grants PB93-0731 and PB93-1163 from DGICYT and SAF 793/94 form CICYT, Ministerio de Educación y Ciencia, Spain. R.T.-S. was supported by fellowships from the F.P.I (AP93), Ministerio de Educación y Ciencia, Spain and V.B.-V. by fellowships from the Ramón Areces Foundation.

References

[1] Carreras, C.W., Santi, D.V., Annu. Rev. Biochem. 64 (1995) 721–762.
[2] Finer-Moore, J.S., Fauman, E.B., Foster, P.G., Perry, K.M., Santi, D.V., Stroud, R.M., J. Mol. Biol. 232 (1993) 1101–1116.
[3] Matthews, D.A., Appelt, K., Oatley, S.J., Xuong, N.H., J. Mol. Biol. 214 (1990) 923–936.
[4] Montfort, W.R., Perry, K.M., Fauman, E.B., Finer, M.J.S., Maley, G.F., Hardy, L., Maley, F., Stroud, R.M., Biochemistry 29 (1990) 6964–6977.
[5] Climie, S., Ruiz-Pérez, L., González-Pacanowska, D., Prapunwattana, P., Cho, S.W., Stroud, R., Santi, D.V., J. Biol. Chem. 265 (1990) 18776–18779.
[6] Hardy, H.W., Nalivaika, E., Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 9725–9729.
[7] Liu, L., Santi, D.V., Biochemistry 31 (1992) 5100–5104.
[8] Liu, L., Santi, D.V., Biochemistry 32 (1993) 9263–9267.
[9] Finer-Moore, J.S., Liu, L., Schafmeister, C.E., Birdsall, T.M., Santi, D.V., Stroud, R.M., Biochemistry 35 (1996) 5125–5136.
[10] Pinter, K., Davisson, V.J., Santi, D.V., DNA 7 (1988) 235–241.
[11] Kealey, J., Santi, D.V., Protein Expression Purif. 4 (1992) 380–385.

[12] Leary, R.P., Beaudette, N.V., Kisliuk, R.L., J. Biol. Chem. 250 (1975) 4864–4868.
[13] Pogolotti, A.L., Danenberg, P.V., Santi, D.V., J. Med. Chem. 29 (1986) 478–482.
[14] García-Fuentes, L., Cámara-Artigas, A., López-Mayorga, O., Barón, C., Biochim. Biophys. Acta 1294 (1996) 83–88.
[15] McKinnon, I.R., Fall, L., Parody-Morreale, A., Gill, S.J., Anal. Biochem. 139 (1984) 134–139.
[16] Freire, E., Mayorga, O.L., Straume, M., Anal. Chem. 62 (1990) 950–959.
[17] García-Fuentes, L., Reche, P., López-Mayorga, O., Santi, D.V., González-Pacanowska, D., Barón, C., Eur. J. Biochem. 232 (1995) 641–645.
[18] García-Fuentes, L., Cámara-Artigas, A., López-Mayorga, O., Barón, C., J. Biol. Chem. 271 (1996) 27569–27574.
[19] Press, W.H., Flannery, B.P., Teukolsky, S.A., Vetterling, W.T. (1988) in: Numerical Recipes in C: The Art of Scientific Computing. Cambridge University Press, New York
[20] Reilly, R.T., Barbour, K.W., Dunlap, R.B., Berger, F.G., Mol. Pharmacol. 48 (1995) 72–79.
[21] Stout, T.J., Stroud, R.M., Structure 4 (1996) 66–77.
[22] Maley, F., Pedersen-Lane, J., Changchien, L., Biochemistry 34 (1995) 1469–1474.
[23] Galivan, J.H., Maley, G.F., Maley, F., Biochemistry 15 (1976) 356–362.
[24] Christensen, J.J., Hansen, L.D., Izatt, R.M., Eds. (1976) Handbook of Proton Ionization Heats and Related Thermodynamic Quantities, John Wiley, New York.
[25] Eftink, M.R., Biltonen, R.L. (1987) in: Hydrolytic Enzymes (Neuberger, A., Brocklehurst, K., Eds.), pp. 333–376, Elsevier, Amsterdam.
[26] Hardy, L.W., Finer-Moore, J.S., Montfort, W.R., Jones, M.O., Santi, D.V., Stroud, R.M., Science 235 (1987) 448–455.
[27] Beaudette, N.V., Langerman, N., Kisliuk, R.L., Arch. Biochem. Biophys. 200 (1980) 410–417.
[28] Wiesenger, H., Hinz, H.J. (1986) in: Thermodynamic Data for Biochemistry and Biotechnology (Hinz, H.J., Ed.), pp. 221–226, Springer-Verlag, Heidelberg.
[29] Mateo, P.L., González, J.F., Barón, C., López-Mayorga, O., Cortijo, M., J. Biol. Chem. 261 (1986) 17067–17072.
[30] Barón, C., González, J.F., Mateo, P.L., Cortijo, M., J. Biol. Chem. 264 (1989) 12872–12878.
[31] Kauzmann, W., Adv. Protein Chem. 14 (1959) 1–63.
[32] Sturtevant, J.M., Proc. Natl. Acad. Sci. USA 74 (1977) 2236–2240.
[33] Livingstone, J.R., Spolar, R.S., Record, M.T. Jr., Biochemistry 30 (1991) 4237–4244.
[34] Privalov, P.L., Makhatadze, G.I., J. Mol. Biol. 213 (1990) 385–391.
[35] Murphy, K.P., Gill, S.J., J. Mol. Biol. 222 (1991) 699–709.
[36] Spolar, R.S., Livingstone, J.R., Record, M.T. Jr., Biochemistry 31 (1992) 3947–3955.
[37] Murphy, K.P., Freire, E., Adv. Protein Chem. 43 (1992) 313–361.
[38] Spolar, R.S., Record, M.T. Jr., Science 263 (1994) 777–784.
[39] Freire, E., Methods in Enzymology 259 (1995) 144–168.