^{31}P NMR STUDIES OF THE BINDING OF ADENOSINE-2′-PHOSPHATE TO *LACTOBACILLUS CASEI* DIHYDROFOLATE REDUCTASE

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1. Introduction

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3) is one of the smallest known dehydrogenases, the enzyme from mammalian and bacterial cells being a monomer with molecular weight in the range 15 000–25 000 (for a review, see [1]). The enzyme is rather specific for NADPH as coenzyme; for example, with the enzyme from *Lactobacillus casei* the K_m for NADH is 170 times that for NADPH and the k_{cat} is less than one tenth that for NADPH [2]. Furthermore, the K_i values for $NADP^+$ and NAD^+ are 1.8 μM and 50 mM, respectively [2]. Clearly the 2′-phosphate group plays a major role in the binding of the coenzyme to the enzyme.

We have already reported studies of the binding of $NADP^+$ and NADPH to the enzyme using ^{31}P NMR spectroscopy [3]; the resonance of the 2′-phosphate was observed to undergo a large downfield shift on binding. In the present paper we describe studies of the binding of adenosine-2′-phosphate, a 'fragment' of the coenzyme, which help to explain the origin of this change in chemical shift on binding and hence provide information on the way in which the 2′-phosphate group interacts with the enzyme.

2. Materials and methods

Dihydrofolate reductase was isolated from *Lactobacillus casei* MTX/R as described previously [2]. The enzyme concentration was determined from its absorbance at 280 nm, by assaying its catalytic activity and by fluorimetric titration with methotrexate [2]. Adenosine-2′-phosphate was obtained from Sigma London Chemical Co., and its concentration determined from its absorbance at 260 nm.

The enzyme was dissolved in D_2O (Norsk Hydro-elektrisk; > 98 atom% D) containing 15 mM bis-Tris, 500 mM KCl, 1 mM EDTA, adjusted to the desired pH* (pH meter reading, uncorrected for the deuterium isotope effect) with 0.1 M KOD or DCl (CIBA (ARL) Ltd., Duxford, Cambs.). The enzyme concentration was 0.8–1.3 mM. Microlitre volumes of a stock solution of adenosine-2′-phosphate (2′-AMP) were added to give concentrations of 0.7–10 mM. In view of the considerable sensitivity of the ^{31}P chemical shifts to pH, care was taken to maintain the pH within ± 0.01 units of the desired value throughout the experiment. All pH measurements were made at the temperature of the NMR experiment, 12 ± 1°C.

^{31}P NMR spectra were obtained at 40.5 MHz in the Fourier transform mode, using a Varian XL-100-15 spectrometer interfaced to a VDM 620-i computer. Sample volumes of 1.5 ml were contained in 12 mm tubes. Up to 100 000 transients were averaged, using an acquisition time of 0.5s and a spectral width of 2 kHz; all spectra were obtained under conditions of proton noise decoupling. Chemical shifts are expressed relative to external inorganic phosphate (pH 8.0). If the exchange of 2′-AMP between the bound and free states is rapid, the chemical shifts as a function of 2′-AMP concentration are described by the equation

$$\delta_{OBS} = \frac{[EL]}{L_T}(\delta_B - \delta_F) + \delta_F \qquad (1)$$

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where δ_{OBS} is the observed chemical shift, δ_B and δ_F the chemical shifts characteristic of the bound and free states, respectively, L_T is the total ligand concentration and $[EL]$ is the concentration of the enzyme–ligand complex; an expression for $[EL]$ in terms of L_T, the enzyme concentration and the binding constant K' is obtained from the mass-action equation. Thus by fitting the data to eq. 1, one can obtain estimates of δ_B and K'. The analysis was performed by a non-linear least squares method, incorporating corrections for dilution and for the concentration-dependence of δ_F (attributable to self-association of 2′-AMP). Between 10 and 15 data points were obtained at each pH.

3. Results

The chemical shift of the ^{31}P resonance of 2′-AMP in the presence of dihydrofolate reductase at pH* 7.0 is shown as a function of 2′-AMP concentration in fig.1. At low concentrations, where a large fraction of the 2′-AMP is bound to the enzyme, the resonance appears substantially downfield of that of free 2′-AMP; as the ligand concentration is increased, its resonance moves upfield towards the position characteristic of free 2′-AMP. This behaviour is characteristic of fast exchange of 2′-AMP between the free and bound states. The curve in fig.1 is the best fit of eq. 1 to the data, calculated with a bound shift, δ_B = 1.21 ppm and a binding constant, K' = 3.6 (±0.5) 10^3 M^{-1}; the excellent fit of the data confirms the applicability of eq. 1.

Similar experiments have been carried out over the pH* range 4.5–7.5 (limited by the stability of the enzyme), and the results are presented in fig.2. Curve A in fig.2 shows the pH-dependence of the ^{31}P chemical shift in the complex (the bound shift, δ_B), and curve B shows the pH-dependence of the binding constant in the form of a Dixon plot [4]. It is clear from curve A

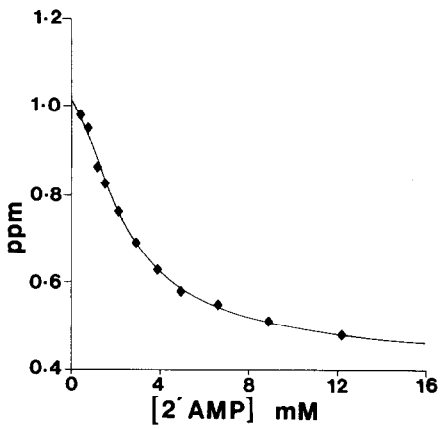

Fig.1. The dependence of the ^{31}P chemical shift of 2′-AMP on the concentration of 2′-AMP in the presence of 1.25 mM dihydrofolate reductase at pH* 7.0. The points are experimental and the line is the best-fit theoretical curve, calculated with the parameters given in the text.

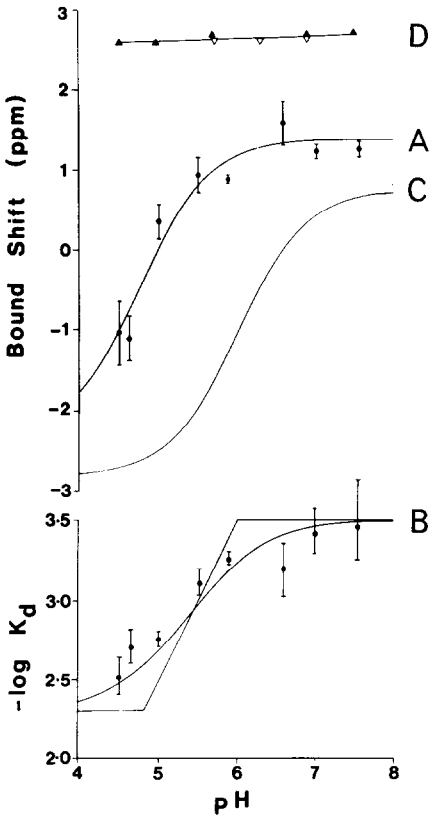

Fig.2. The pH-dependence of 2′-AMP binding to dihydrofolate reductase. (A) The ^{31}P chemical shift in the 2′-AMP: enzyme complex. (B) The dissociation constant of the complex. (C) The pH-dependence of the ^{31}P chemical shift of free 2′-AMP. (D) The 2′-phosphate ^{31}P chemical shifts of NADPH (open symbols) and $NADP^+$ (filled symbols) in their complexes with the enzyme (from [3]). The curves in A and B were calculated with the parameters given in the text.

that the 2′-phosphate group of 2′-AMP can ionise when it is bound to the enzyme, that is, both the monoanion and dianion can bind. Comparison of curve A, the titration curve of the 2′-phosphate group in the complex, with curve C, the corresponding titration curve of free 2′-AMP, indicates that the pK of the phosphate group is significantly lowered on binding to the enzyme. Combined analysis of the chemical shift and binding constant data yields a pK value for bound 2′-AMP of 4.8 ± 0.2, compared to 6.0 ± 0.05 for free 2′-AMP under these conditions. The *curves* in A and B of fig.2 are theoretical curves calculated with these pK values. Within experimental error, this change in pK of the phosphate group on binding adequately explains the pH dependence of the binding constant (curve B), indicating little if any change in the pK values of groups on the enzyme titrating in the pH range 5–7 when 2′-AMP binds.

The decrease of 1.2 units in the pK of the phosphate group on binding to the enzyme shows that the dianionic form of 2′-AMP binds 16-fold more tightly than the monoanionic form. The binding constants obtained from the data in fig.2 are 3.6 (± 0.2) 10^3 M^{-1} for the dianion and 2.3 (± 1) 10^2 M^{-1} for the monoanion.

For comparison, curve D in Figure 2 shows the ^{31}P chemical shift of the 2′-phosphate group of $NADP^+$ and NADPH in their complexes with the enzyme, taken from [3].

4. Discussion

The equilibrium constant for 2′-AMP binding at pH 7.0, 12°C, is 3.6 10^3 M^{-1} ($\Delta G^{o\prime}$ = –4.6 kcal/mol), while under the same conditions that for $NADP^+$ is 4.5 10^4 M^{-1} ($\Delta G^{o\prime}$ = –6.0 kcal/mol; B. Birdsall and S. Dunn, unpublished work). In contrast, the binding of 5′AMP is too weak to be detectable by NMR. The experiments with coenzyme 'fragments' thus confirm the importance of the 2′-phosphate group for coenzyme binding to dihydrofolate reductase. Indeed, the simple comparison of binding constants suggests that three-quarters of the binding energy of $NADP^+$ is attributable to the 2′-AMP fragment; however, as will be seen, this comparison is not quantitatively valid.

The pH-dependence of the ^{31}P chemical shift of bound 2′-AMP and that of its binding constant show clearly that this compound binds more tightly to the enzyme when its phosphate group is in the dianionic, rather than the monoanionic, charge state. In free energy terms the preference for the dianionic form is approximately 1.6 kcal/mol. The observation that the ^{31}P chemical shift of the 2′-phosphate group of $NADP^+$ or NADPH bound to the enzyme is pH-independent over the range 4.5–7.5 [3] indicates that this group must have a pK outside the range pH 3.5–8.5 in these complexes. The ^{31}P chemical shift of the 2′-phosphate resonance of $NADP^+$ and NADPH when bound to the enzyme is much closer to that characteristic of the dianionic form than to that of the monoanionic form [3]. Taken together with the results for 2′-AMP, this clearly indicates that the 2′-phosphate group of $NADP^+$ and NADPH must also be in the dianionic charge state when bound. The limit noted above on the pK of the phosphate in the coenzyme complexes shows that for $NADP^+$ and NADPH the preference for the dianionic form of the 2′-phosphate must be at least 300-fold, or 3.2 kcal/mol – at least double that found for 2′-AMP.

Since these ligands bind preferentially to the enzyme in the dianionic form, the change in the ^{31}P chemical shift of the 2′-phosphate group on binding will be made up of two parts. One contribution will arise simply from the increased fraction of the ligand in the dianionic form in the complex, and the other, the 'extra' bound shift (given by the difference between the chemical shifts of the *dianionic* molecule in the bound and free states), from changes in the magnetic and/or electronic environment of the 2′-phosphate group on binding to the enzyme. This second contribution amounts to 0.5 ppm for 2′-AMP and 2.0 ppm for $NADP^+$ and NADPH (both shifts to low field). The phosphorus nucleus in a phosphate group is to some extent 'insulated' from its environment by the surrounding oxygen atoms. Thus, for example, an aromatic ring can not approach closer than about 4.9 Å, leading to a *maximum* downfield 'ring-current'shift of ~ 0.2 ppm. Similarly, proximity of charged groups to a phosphate has little effect beyond that attributable to the change in pK of the phosphate [5,6]. The observed preference for the dianionic form of the phosphate suggests an interaction with a pair of positive charges on the enzyme, or, perhaps more probably, with the 'bidentate'

positively charged guanidino group of an arginine residue. (Two arginine residues of dihydrofolate reductase are protected from reaction with phenylglyoxal in the presence of NADPH [7], but their specific role in coenzyme binding remains to be established.) However, it seems unlikely that the observed 'extra' downfield shift of the 2′-phosphate resonance can be ascribed simply to the proximity of these charged group(s). Thus, when dianionic cytidine 3′-monophosphate binds to pancreatic ribonuclease A, its ^{31}P resonance is shifted upfield by less than 0.3 ppm [8], in spite of the highly positive local environment, and the formation of hydrogen-bonds with the enzyme [9]. Recently Gorenstein [5,10,11] has proposed that the ^{31}P chemical shifts of phosphates are extremely sensitive to very small changes in the O–P–O bond angle, and the consequent rehybridisation. Such a slight distortion of the phosphate group, which might be required for optimal interaction with the enzyme, is an attractive explanation for the 'extra' bound shift observed on the binding of 2′-AMP, $NADP^+$ and NADPH to dihydrofolate reductase. (A similar explanation has recently been proposed [12] for the much larger downfield shift observed for the covalently bound phosphate in alkaline phosphatase [12–14].) On this hypothesis, both the increased preference for the dianion and the increase in the 'extra' bound shift on going from 2′-AMP to $NADP^+$ would be ascribed to small differences in the geometric relationship between the 2′-phosphate and the positively charged group(s) with which it interacts.

The clear differences in the behaviour of the 2′-phosphate group in 2′-AMP and $NADP^+$ show that the environment of this group is significantly different in the complex of the enzyme with the 'fragment' as compared to that with the complete coenzyme. This in turn means that the binding energy of 2′-AMP may not be an accurate measure of the contribution of this part of the molecule to the binding of $NADP^+$. Experiments with the 'fragments' adenosine 2′,5′-diphosphate and 2′-phosphoadenosine-5′-diphosphoribose (our unpublished work) suggest that the environment of the 2′-phosphate group progressively approaches that seen for $NADP^+$ as the resemblance of the 'fragment' to the coenzyme increases. At present we do not know whether these effects simply reflect small changes in the orientation of the 2′-AMP moiety of the coenzymes in its binding site, or whether they reflect conformational differences, although there is clear evidence from NMR and kinetic work (S. Dunn, R. W. King, B. Birdsall, J. G. Batchelor G. C. K. Roberts, J. Feeney and A. S. V. Burgen, unpublished work) that a conformational change does accompany the binding of $NADP^+$ and its larger 'fragments'. The progressive changes in the environment of the 2′-phosphate group appear to be complete at the level of $NADP^+$; the thousand-fold increase in binding constant observed on reduction of the nicotinamide ring is not accompanied by any change in the environment of the 2′-phosphate [3] (cf. fig.2).

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