Binding of Fluoride by Yeast Enolase†

Frank J. Bunick‡ and Shelby Kashket*

ABSTRACT: The kinetics of fluoride binding by yeast enolase have been examined by direct measurement of equilibrium fluoride (F) concentrations with an ion-specific electrode. Mg^{2+} and inorganic phosphate (P_i) affect the binding of F, and evidence is presented for an ordered binding mechanism in which phosphate and F interact with conformational and catalytic Mg^{2+} species, with the overall formation of a quaternary complex. A maximum of four atoms of F were bound per enzyme dimer, and the data point to the nonequivalent binding of pairs of F atoms. The dissociation constants were 5.0×10^{-4} M and 8.2×10^{-5} M for the first and second pairs of F atoms, respectively. The first pair of binding sites was filled when the ratio of phosphate ions/enolase dimer exceeded 2 and appeared to involve the pair of conformational Mg^{2+} ions. The binding of the second pair of F atoms followed the binding of catalytic Mg^{2+} in the presence of P_i and, furthermore, appeared to exhibit positive cooperativity with respect to F. The data suggest, also, that the binding of P_i may involve sequential addition of P_i pairs to the different Mg^{2+} species on the enzyme. F binding was at a maximum between pH 5.5 and pH 6.0, consistent with an involvement of the monovalent form of P_i. In the absence of added P_i, $(MgF)^+$ appeared to be the preferred ligand. Addition of the enzyme substrate 2-phosphoglycerate led to the release of bound F. These findings are consistent with the known patterns of inhibition of enzymatic activity by F.

Enolase catalyzes the elimination of H_2O from 2-phosphoglycerate to yield phosphoenolpyruvate. The enzyme is composed of two identical subunits (Brewer & Weber, 1968; Brewer, 1981) that require two Mg^{2+} ions for dimerization and a second pair of Mg^{2+} ions for activation to enzymatic competence (Hanlon & Westhead, 1969; Brewer, 1974; Faller et al., 1977). Elevated levels of Mg^{2+} lead to inhibition of enzymatic activity (Wang & Himoe, 1974).

The inhibition of the enzyme by fluoride has been known for many years, and Warburg & Christian (1941) suggested that the inhibitory effect is due to the formation of a magnesium–fluoride–phosphate complex. More recently, Cimasoni (1972) showed that the enzyme from rabbit muscle was only weakly affected by F[1] alone and that, in the presence of P_i, the inhibition was strong and competitive with respect to the substrate. Ruth et al. (1970) found no inhibition of salmon enolase by F alone. Wang & Himoe (1974) confirmed these findings and proposed that P_i and F bind cooperatively to the enzyme and, more specifically, that F is coordinated to the enzyme-bound Mg.

Direct measurements of F binding to enolase were not made and earlier proposals concerning the mechanisms of inhibition were deductive in nature. Recently, Maurer & Nowak (1981) applied nuclear magnetic resonance and light spectrographic methodologies to the problems of ligand binding to enolase and obtained data that indicate a binding of F to enzyme-bound Mn. In the present studies we have made use of an ion-specific electrode to follow F binding to enolase directly and have obtained data that appear to reflect the interactions of F with Mg^{2+} and P_i within the enzyme matrix. These findings have been presented in a preliminary form (Kashket & Bunick, 1979).

Materials and Methods

Yeast enolase (phosphopyruvate hydratase, 2-phospho-D-glycerate hydro-lyase EC 4.2.1.11) was obtained from Sigma Chemical Co., St. Louis, MO (type III, approximately 60 units/mg of protein), as a lyophilized powder containing buffer salts. Polyacrylamide gel electrophoresis at pH 8.3, in the presence of 2 mM $MgSO_4$, revealed one major and two minor protein bands [cf. Porcelli et al. (1978)], all of which were enzymatically active. No major contaminant could be detected. D(+)-2-Phosphoglycerate was obtained as the sodium salt from Sigma Chemical Co. All other salts were reagent grade. Deionized water was used throughout.

Fluoride ion activities were measured directly with a F ion specific electrode (Orion Research, Inc., Cambridge, MA). Yeast enolase (about 35 mg) was dissolved in 6 mL of a buffer consisting of 0.05 M Tris-HCl, pH 7.5, and 0.10 M KCl, plus $MgSO_4$ and potassium phosphate, as indicated, and dialyzed overnight at 4 °C against 2 L of the same buffer. In the early experiments involving titrations with $MgSO_4$, NaF was included in the dialysis system, and it was found that F was bound to the enzyme under these conditions and could be released by boiling the dialyzed preparation. For all subsequent experiments, therefore, the overnight dialysis was carried out without added F, and the F was added only at the start of the titration experiment.

A total of 5 mL of the dialyzed enzyme preparation was titrated by adding increasing volumes of the desired titrant, with constant stirring, and measuring the F ion activity after each addition. Each addition was in the range of 5–20 μL, and the total volume that was added during an experiment was no more than 350 μL, so that the increase in the total volume of the system was, at maximum, 6.5%. Controls were prepared by titrating 5.0 mL of buffer with exactly the same volumes, and in the same sequence, as were used for the samples containing the enzyme. The binding of F was calculated as the difference between the F concentrations in the experimental and control samples for each addition of titrant. In the controls for the experiments involving titration with NaF at the higher concentrations of P_i (2 or 15 mM P_i), the measured F ion activity was lower than the theoretical, especially at the very

† From the Biochemistry Department, Forsyth Dental Center, Boston, Massachusetts 02115. *Received January 25, 1982; revised manuscript received April 16, 1982.* This work was entirely supported by the National Institute of Dental Research, National Institutes of Health (Research Grants DE-03917 and DE-03430).

‡ Present address: Food Science and Nutrition Department, University of Massachusetts, Amherst, MA 01002.

[1] Abbreviations: F, fluoride which, unless specified, refers to both F^- and HF; F_b, enzyme-bound F; F_f, free F at equilibrium; PGA, D-2-phosphoglycerate; P_i, orthophosphate; Tris, tris(hydroxymethyl)aminomethane.

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low concentrations of added F. This was due, apparently, to the formation of complexes between the ionic species. As a result, the F binding curves that were obtained at the high P_i concentrations represent enzymatic binding plus a small amount of complex formation. It is important to note, however, that the final curves for F binding (Figure 2) were altered only slightly by this treatment.

The experiments involving the titration with acid were carried out by adding 5–20-μL aliquots of 0.024 N HCl to a total volume of 500 μL. No more than 300 μL was needed, however, to drop the pH to about 4. All further additions of HCl served mainly to convert F^- to HF ($pK = 3.24$) so that, below pH 4, the F^- ion activity decreased rapidly in both the control and enzyme-containing samples. Because of the differences in the buffering capacities of the two samples, the addition of equal volumes of HCl gave different equilibrium pH values. Consequently, so that one could calculate the binding of F, the curves relating millivolts and pH were drawn as shown in the inset to Figure 5, and new millivolt values for the control and the enzyme-containing samples were read from the curves at intervals of 0.25 pH unit. The corresponding F concentrations were calculated from these values, and the differences were plotted as shown in Figure 5.

The ionic strength of the Tris–KCl system was 0.15 M (with 15 mM P_i present this value rose to 0.20 M), and the activity coefficient was about 73% (Orion Research, Inc., Cambridge, MA). All experiments were carried out at 25 °C.

Results

Preliminary experiments showed that the concentration of F^- ions was reduced following the incubation of NaF with dialyzed yeast enolase. The addition of increasing amounts of enzyme protein led to progressively lower concentrations of F in the medium, consistent with the binding of F by the enzyme. The ΔF increased linearly from about 0.1×10^{-4} to 1.85×10^{-4} M as the enzyme concentration was increased from 0.5 to 8.0 mg/mL. As a consequence of these experiments, most of the subsequent studies were carried out with 5–6 mg of enolase protein/mL.

Effect of Mg^{2+}. Enolase was dialyzed against buffer containing 10^{-6} M $MgSO_4$ and 6.6×10^{-4} M NaF and then titrated with increasing amounts of $MgSO_4$. The resulting adsorption isotherm is shown in Figure 1A. It can be seen that, in the absence of added P_i, F binding was poor and increased only after the concentration of $MgSO_4$ had reached about 8×10^{-4} M. When enzyme was omitted and buffer plus NaF was titrated with the $MgSO_4$, a similar curve was obtained (Figure 1A), but the reduction in free F was less than when the enzyme was present. The latter curve was consistent with the formation of $(MgF)^+$, and indeed, the association constant for this species, when calculated from the data, was 20.6, almost in perfect agreement with the published value of 20.0 (Connick & Tsao, 1954). The difference between the two curves was attributed to binding by enolase and pointed to the binding of $(MgF)^+$ under these conditions.

The addition of 15 mM P_i to the system led to an increased binding of F to the enzyme (Figure 1B). Binding was curvilinear with respect to the concentration of $MgSO_4$, and a maximum was achieved at $(1–2) \times 10^{-3}$ M added $MgSO_4$. The shift in the adsorption isotherm to the left, relative to the curve shown in Figure 1A, indicated a considerable increase in the affinity of the enolase for F. Further additions of $MgSO_4$ beyond 2×10^{-3} M led to a reduction in the amount of F bound. The reduction was evident in spite of the fact that these conditions favored the formation of $(MgF)^+$ and should have led to an apparent "increase" in F binding.

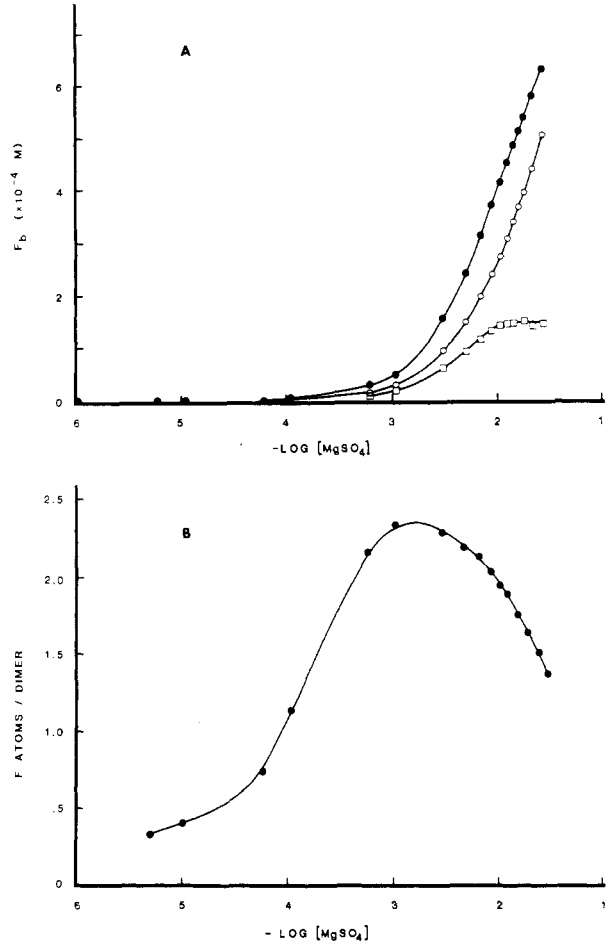


FIGURE 1: Binding of F to yeast enolase as a function of the concentration of added $MgSO_4$. Yeast enolase (9.6×10^{-5} M) in 5 mL of 0.05 M Tris-HCl, pH 7.5, 0.10 M KCl, and 0.66 mM NaF was titrated with incremental additions (5 or 20 μL) of 5×10^{-3}, 5×10^{-2}, and 5×10^{-1} M $MgSO_4$ solutions to give the indicated final concentrations. The total volume added was 350 μL. F^- ion activity was determined at 25 °C with a F-specific electrode. (A) In the absence of added P_i; (B) with 15 mM P_i present. (●) Complete system; (○) system without enzyme; (□) difference between the enolase-containing system and the blank without enolase.

The maximum amount of F that was bound was equivalent to 2.3 atoms/dimer of enolase [88 000 daltons, according to Mann et al. (1970)]. Larger values were obtained in subsequent experiments but only when F was omitted from the preliminary dialysis of the enzyme. This was consistent with the binding of F during dialysis, even at the existing very low concentrations of Mg^{2+}. In the present experiments the enolase was dialyzed against buffer that contained F, P_i, and 10^{-6} M $MgSO_4$. When an aliquot of the dialyzed enzyme was boiled, it was found to release F that was equivalent to 0.7 atom/enolase dimer, in agreement with the findings of Hanlon & Westhead (1969) and Faller et al. (1977). Thus, after the first few additions of Mg^{2+} (Figure 1B), the total F bound was 0.3 plus 0.7, or 1.0, atom/dimer while, at 1.1 mM added $MgSO_4$, the total was 2.3 plus 0.7, or 3.0, atoms/dimer.

F Concentration. The addition of increasing amounts of F led to an increased binding of the anion by enolase (Figure 2). Examination of the adsorption isotherms showed that, under all conditions, binding increased to a maximum and that the maximum was dependent on the concentration of P_i present. $MgSO_4$ was present in a concentration of 2 mM. In a separate experiment (not shown) it was found that F binding was greatly reduced in the presence of 5×10^{-5} M $MgSO_4$ and 2 mM P_i, in agreement with the data shown in Figure 1B.

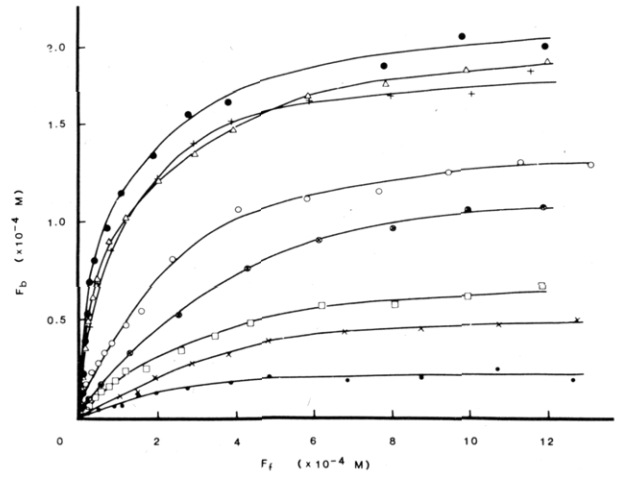


FIGURE 2: F binding with increasing concentrations of F and P_i. Adsorption isotherms. Enolase (5.2×10^{-5} M) was incubated in 5 mL of Tris–KCl, pH 7.5, 2 mM $MgSO_4$, and the indicated concentrations of P_i. Titration was with 5×10^{-2} M NaF. For definition of the symbols, see Figure 4.

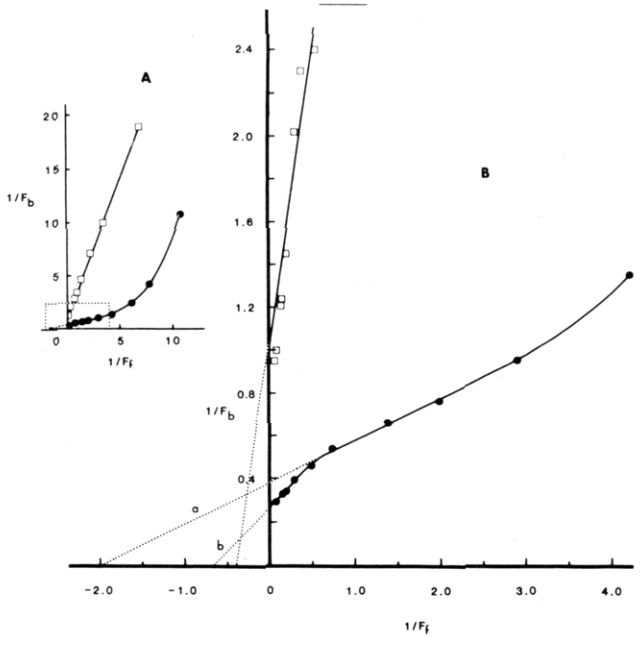


FIGURE 3: F binding with increasing concentrations of F and P_i. Same conditions as for Figure 2. (A) Double-reciprocal plots of the data for the experiments with 0.05 and 15 mM P_i. (B) Expansion of the plot from the section demarcated by the dashed line in (A). Slopes and extrapolations were calculated by linear regression analysis. For definition of the symbols, see Figure 4.

The curve was reduced to one resembling that shown in Figure 2 for 2 mM $MgSO_4$ and 0.025 mM P_i. Essentially no F was bound if the titration of the enzyme was carried out in the absence of added P_i, yet addition of a pulse of P_i at the end of the titration experiment (i.e., at 1.6 mM NaF) resulted in the sudden binding of 2.38 F atoms/enolase dimer.

In a separate series of experiments, enolase was titrated with increasing concentrations of P_i, in the presence of 1.32 mM NaF and 2 mM $MgSO_4$. F binding increased from about 10^{-5} M P_i and reached a plateau at about 10^{-3} M P_i (P_i/enolase dimer = 20). The broad range of effectiveness of P_i was consistent with the data shown in Figure 2, and indeed, data from the latter figure could be superimposed on the curves that were obtained after the titrations with P_i.

The F binding data from the experiments with 0.05 and 15 mM P_i were plotted according to Langmuir (1918), giving the

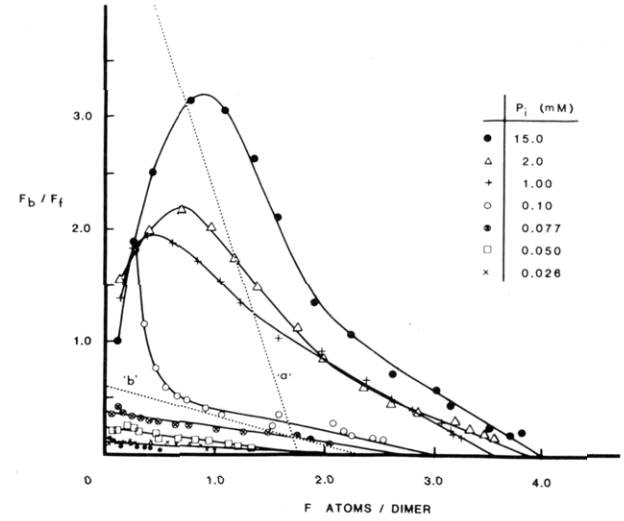


FIGURE 4: F binding with increasing concentrations of F and P_i. Same conditions as for Figure 2. The plot is according to Scatchard (1949). Lines a and b [calculated according to Pennock (1973)] are shown for the 15 mM P_i system. Symbols are the same for Figures 2 and 3.

Table I: Calculated Values for K_D and n_{app}

$[P_i]$ (mM)	$[P_i]$/ [dimer]	K_D (M) a[a]	K_D (M) b[a]	n_{app} a	n_{app} b
15.0	300	1.7×10^{-5}	1.9×10^{-4}	1.7	2.1
2.0	40	3.1×10^{-5}	2.0×10^{-4}	1.6	1.9
1.0	20	4.3×10^{-5}	2.1×10^{-4}	1.6	1.9
0.1	2.05	1.1×10^{-5}	4.2×10^{-4}	0.4	2.3
0.08	1.5		3.8×10^{-4}		2.5
0.05	1.0		3.7×10^{-4}		1.7
0.026	0.5		5.4×10^{-4}		1.7

[a] Lines a and b from curves as shown for 15 mM P_i in Figure 4. Slopes for lines that were obtained at the lower concentrations of P_i were calculated by linear regression analysis.

curves shown in Figure 3A. At the lower P_i concentration, a straight line was obtained with intercepts that gave a K_D of 1.3×10^{-4} M and a maximum binding of 5.3×10^{-5} M. The results with 15 mM P_i gave a line that curved upward at higher values of $1/F_f$, suggesting that cooperative effects applied under these conditions. A plot of the data from the low $1/F_f$ region in an expanded form (Figure 3B) revealed two distinct slopes in this segment of the curve. When the slopes were extrapolated, lines a and b were obtained, and K_D values of 2.6×10^{-5} M and 8.1×10^{-5} M and binding maxima of 1.4×10^{-4} M and 2.0×10^{-4} M, respectively, were calculated.

A plot of F_b/F_f vs. F_f, according to Scatchard (1949), gave a family of curves with configurations that changed progressively with increasing concentrations of P_i (Figure 4). At low P_i concentrations, the lines appeared to be straight and gave intercepts on the abscissa that corresponded to approximately 2 F atoms/enolase dimer. When the P_i concentration was increased, the number of apparent binding sites (n_{app}) increased, and at 0.1 mM P_i (when the ratio of P_i/enolase dimer was 2.05), the line became curvilinear. Above this concentration of P_i the curves remained curvilinear but acquired, in addition, a positive slope and exhibited maxima when the number of F atoms per enolase dimer was less than 1. At a P_i concentration of 1 mM (P_i/dimer = 20) the maximum F_b/F_f was about 2.0, while at a P_i concentration of 15 mM (P_i/dimer = 300) the maximum was 3.2. Analyses of the downward sloping segments of the curves were carried out by the method of Pennock (1973). The example shown in Figure

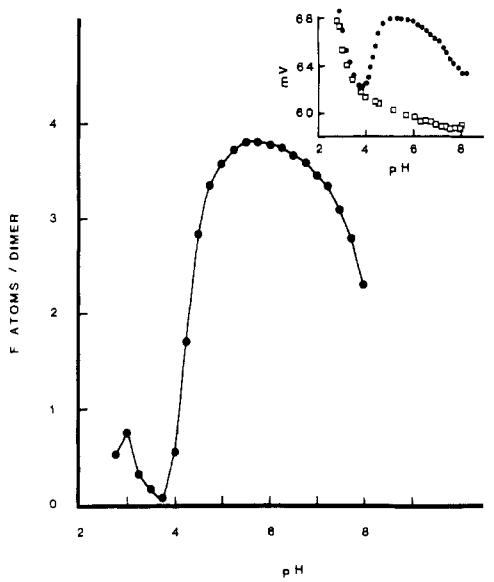


FIGURE 5: Effect of pH on binding of F by enolase. The enzyme (6.2×10^{-5} M) in 5 mL of 0.165 M KCl (no Tris-HCl), 0.79 mM NaF, 2.0 mM $MgSO_4$, and 2 mM K_2HPO_4, pH 8.02, was titrated with 0.024 N HCl. The inset shows original data.

4 (dotted lines) for 15 mM P_i gave dissociation constants for slopes of a and b of 1.7×10^{-5} M (K_D^a) and 1.9×10^{-4} M (K_D^b), respectively. The calculated constants for the remaining curves are presented in Table I. It can be seen from the table that the K_D values decreased progressively as the concentrations of P_i increased. The exception was the K_D^a for the curve that was obtained with 0.1 mM P_i. This curve appeared to reflect a transition from the simpler to the more complex mechanism of F binding.

The slopes and intercepts of the b components of the curves obtained in the high P_i concentration systems were similar to those for the simpler lines that were obtained at the lower P_i concentrations. Thus, at 2.6×10^{-5} M P_i the K_D was found to be 5.4×10^{-4} M, while linear extrapolation of all K_D^b values to zero P_i concentration gave a K_D of about 5.0×10^{-4} M. The K_D values for the a component of the high P_i concentration containing systems extrapolated to about 8.2×10^{-5} M.

Table I also contains the calculated values for the apparent number of F binding sites for each line. The values for n_{app}^b were close to 2.0 for all concentrations of P_i except 0.08 and 0.10 mM, when the values were 2.5 and 2.3, respectively. The values for n_{app}^a were about 1.7, except for the system containing 0.1 mM P_i, where the n_{app} was 0.4.

Effect of Hydrogen Ion Concentration. Titration of the enzyme with increasing concentrations of HCl showed that enzyme-bound F was at a maximum at about pH 6 (Figure 5). In the presence of 2 mM each of $MgSO_4$ and P_i, and 7.9×10^{-4} M NaF, the binding increased as the pH rose from 3.75 to 5.5 and then decreased as the pH was raised further from 6.0 to 8.2. At the maximum, the number of F atoms per enolase dimer was 3.8, a value that was consistent with the extrapolated value of 4 for the total number of binding sites per enzyme molecule (Figure 4; Table I). It is interesting that the number of F atoms per enolase dimer was only 3.1 at pH 7.5, the pH that has been used generally for the assay of enolase, as well as for the present experiments on F binding. When the $MgSO_4$ concentration was kept at 0.05 rather than 2 mM, the maximum number of F atoms per enolase dimer was only about 2.2 at pH 6.25 and 0.9 at pH 7.5. Titrations in the reverse direction gave essentially the same results. The full interpretation of these data is difficult, especially in view of the known reversible denaturation of enolase at low pH (Westhead, 1964). The reduction in F binding above pH 6, however, suggests that the monovalent form of P_i is involved in the binding process.

Release of Bound F by PGA. Inhibition of enolase by F in the presence of P_i has been shown to be competitive with respect to the substrate PGA (Cimasoni, 1972; Wang & Himoe, 1974). Furthermore, Wang & Himoe (1974), Stubbe & Abeles (1980), and Nowak & Maurer (1981) suggested that F may compete with the –OH group of PGA for bound Mg^{2+}. Consequently, the F binding experiments were repeated (with concentrations of $MgSO_4$ and P_i of 2 and 5 mM, respectively), but after electrode equilibrium was attained, PGA was added to a final concentration of 4 mM. It was found that F was released from the bound form. In one such experiment, the initial NaF concentration was 0.105 mM, and after the addition of the enzyme, this was reduced to 0.043 mM. Addition of PGA led to an increase in the measurable F to 0.087 mM. Thus, 70.2% of the F that was initially bound was released after the addition of the substrate. In the absence of added P_i the addition of substrate resulted in the release of all of the bound F. It is important to note that the PGA and Mg^{2+} concentrations were such that all of the metal ions could have been chelated (K_a for Mg–PGA = 281). However, P_i induced an increase in the affinity of the enzyme for Mg^{2+} and favored the retention of bound F.

F Binding by Streptococcal Enolases. Studies were carried out with enolases that were purified from *Streptococcus salivarius* strain 25975 and *Streptococcus mutans* strain IB 1600 (Bunick & Kashket, 1981), microorganisms that have been shown to be strongly and weaky inhibited, respectively, by low concentrations of F (Kashket et al., 1977). Both enzymes exhibited binding curves that were similar to those shown in Figure 2. With 2 mM $MgSO_4$ and 15 mM P_i, the K_D^a for the former enzyme was 6.3×10^{-6} M and the K_D^b was 2.0×10^{-4} M. The amounts of enolase from the *S. mutans* strain that were available were too small to permit accurate determinations of the first dissociation constant but gave a K_D^b of 4.0×10^{-4} M.

Discussion

There was, at best, very little F bound to enolase at low levels of Mg^{2+} when no P_i was added (Figure 1A). At the concentrations of Mg^{2+} that applied in such experiments the conformational Mg^{2+} probably was the only species that was present on the enzyme (i.e., $K_1^{Mg} < K_2^{Mg} = 2 \times 10^{-7}$ M; Faller et al., 1977). As Mg^{2+} was added to the system, both conformational sites were likely to become filled, but it is uncertain whether the catalytic sites became occupied with Mg since substrate was not present in most of these experiments (Hanlon & Westhead, 1969; Faller et al., 1977). In spite of this, as the Mg^{2+} concentration was increased beyond the millimolar level, F binding became apparent. At these concentrations, it could be shown that the activity of F^- decreased [presumably through the formation of $(MgF)^+$]. The coincidence of these two events suggested that F became bound to the enzyme as $(MgF)^+$. Evidently, $(MgF)^+$ was an acceptable ligand even in the absence of substrate.

The addition of P_i changed the conditions considerably and apparently made possible the binding of F to both types of Mg binding sites on the enzyme. Thus, F became bound during dialysis of enolase in buffer containing F and P_i but no added Mg^{2+}, since the enzyme probably contained conformational Mg^{2+} even under these conditions. After the addition of about 1×10^{-5} M $MgSO_4$ a condition existed, presumably, where only conformational Mg^{2+} was present and catalytic Mg^{2+} was

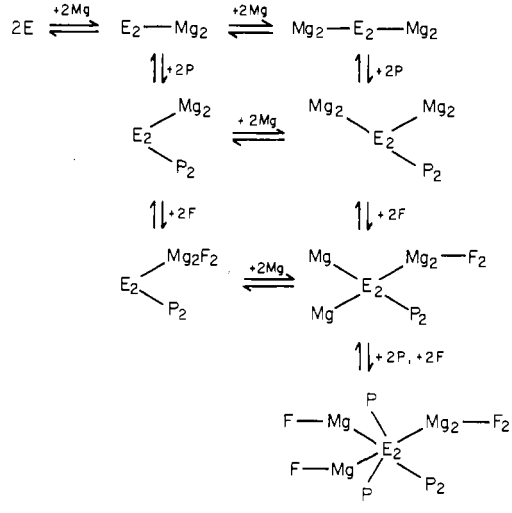
not yet available on the enzyme ($K_{3,4}^{Mg} = 5 \times 10^{-5}$ M; Faller et al., 1977), and the enzyme bound a total of 1.1 F atoms/dimer. With higher concentrations of Mg^{2+}, the number of F atoms per dimer rose to about 3, and it appeared from this that the second pair of Mg^{2+} ions could add to the catalytic sites of the enzyme in the presence of P_i and that the F followed the cations into the sites. The parallel between the concentrations of $MgSO_4$ that were needed to affect F binding and the published values for the dissociation constants for Mg^{2+} makes it reasonable to conclude that F binding was to the catalytic sites on the enzyme rather than to some other, nonspecific locations. This conclusion is in agreement with that of Maurer & Nowak (1981), who reported that F probably binds directly to enolase-associated metal ligand and that F binding is enhanced by P_i.

The experiments on the effects of increasing concentrations of F and P_i on the binding of F (Figures 2–4) permitted a further analysis to be made of the mechanisms that are involved. At low P_i concentrations (i.e., when the number of P_i ions per enolase dimer was 1 or less) only 2 atoms of F were bound per dimer. Since a similar degree of binding was obtained with 5×10^{-5} M $MgSO_4$ and high P_i concentration, it appears that the binding at low P_i involved the conformational Mg. When the P_i/dimer ratio was 1.5, the number of binding sites increased to 2.5, and when the ratio was 2.05 (i.e., the P_i concentration was equimolar with the enzyme concentration), the F_b/F_f vs. F_b curve became distinctly biphasic. Thus, F binding appeared to "spill over" to a second site, probably to the second pair of bound Mg^{2+} ions, and a second K_D could be determined (Table I). It is significant that the n_{app} for the new site was only 0.4, and it appeared that only one of the second pair of binding sites might be operational.

For the present argument it is assumed that the biphasic nature of the binding reflects F binding at two independent pairs of sites rather than a negative cooperativity between F binding sites (Norby et al., 1980). This assumption is based mainly on the existence of distinct pairs of conformational and catalytic Mg^{2+} binding sites on the enzyme, sites that are considered as capable of binding F. The finding that, as the P_i/enolase dimer ratio was raised to 20 or higher, the total number of F atoms per dimer approached what appeared to be a maximum of 4 supports the proposal that two pairs of sites are involved in the binding of F. The relatively abrupt transition from straight lines to biphasic curves as the P_i/dimer ratio was raised to 2 was consistent with the sudden involvement of the second of the two pairs of F binding sites.

At the elevated concentrations of P_i the patterns of binding changed further. Thus, the curves in the double-reciprocal plot exhibited a marked upward curvature at high values of $1/F_b$ (Figure 3), and the curves in the Scatchard plots (Figure 4) acquired a positively sloped segment at low values of F_b (i.e., number of F atoms per dimer). A simple interpretation of the upward curvature is that the addition of both F atoms of the second pair was subject to positive cooperativity (Segel, 1975). This conclusion is in agreement with the proposal of positive cooperativity for F that was made by Maurer & Nowak (1981), although it is not certain whether the cooperativity discerned in their experiments involved the interactions between the pairs of F atoms or the presently described interaction between the F atoms of the second pair. In the latter study, Mn^{2+} was used rather than Mg^{2+}, the concentrations of P_i were relatively low, and the concentrations of F generally were much higher than those that were used in the present experiments, and it is difficult to compare their results directly with ours. The differences in experimental conditions as well as in methodologies may explain, also, why Maurer and Nowak proposed a random addition of both F and P_i to the enzyme. Our findings point to an ordered addition of F and suggest, as well, a sequential addition of P_i to sites 3 and 4. Thus, the observed dependence of K_D on P_i (Table I) is consistent with an ordered addition of P_i to the enzyme (Segel, 1975). Admittedly, though, the effect may be due to increasing ionic strength (with progressive additions of P_i) on F binding (Lent et al., 1976) and may not reflect the nature of the P_i binding mechanisms.

In summary, it is proposed that optimal F binding to enolase is dependent on the presence of Mg^{2+} and P_i. The addition of Mg^{2+} ions to the conformational sites is followed, at low concentrations of P_i, by a pair of P_i ions and subsequently by a pair of F^- ions. As the concentration of added P_i increases, and before the first pair of sites becomes fully occupied, a single P_i ion adds to one of the catalytic Mg^{2+} sites, and this enables the third F^- ion to bind. The addition of the fourth P_i permits the final F^- ion to approach. However, binding of this F^- ion is affected by the presence of the third F, and a positive cooperative interaction between the two sites is observed. The mechanism is depicted in the following scheme.



It is important to note that F is shown to bind to Mg and that P_i is shown to bind to the enzyme at a site that is removed from the metal ligand, in agreement with the suggestions of Maurer & Nowak (1981) and Brewer (1981).

The data and the mechanism that have been put forward are consistent with the complex patterns of inhibition of enzymatic activity by F that have been reported in the literature. However, a fuller understanding of the mechanisms of F binding by enolase will require further studies in which substrates of the enzyme, or their transition-state analogues (Spring & Wold, 1971), are present and taken into consideration.

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Evidence on the Existence of a Purine Ligand Induced Conformational Change in the Active Site of Bovine Pancreatic Ribonuclease A Studied by Proton Nuclear Magnetic Resonance Spectroscopy†

Carles Arûs, Livio Paolillo, Rafel Llorens, Roberto Napolitano, and Claudi M. Cuchillo*

ABSTRACT: The titration curves of the C-2 histidine protons of RNase A and of derivative II—a covalent derivative obtained by reaction of the enzyme with the halogenated nucleotide 9-β-D-ribofuranosyl-6-chloropurine 5′-phosphate—in the presence of a number of purine nucleosides, nucleoside monophosphates, and nucleoside diphosphates were studied by means of proton nuclear magnetic resonance at 270 MHz. The examination of the perturbations found on the chemical shifts and p*K*s of the C-2 protons of His-12, -48, and -119 are consistent with the following conclusions: (1) The interaction of adenosine in the primary purine binding site of the enzyme (B_2R_2) induces a conformational change in the active center of the enzyme [for the nomenclature of the RNase A binding subsites, see Parés et al. [Parés, X., Llorens, R., Arûs, C., & Cuchillo, C. M. (1980) *Eur. J. Biochem. 105*, 571–579]]. (2) The phosphate moiety of the ligands, independently of its position, probably acts as a general carrier of the nucleotide to the active center, while the substituents of the base are the generators of the specificity of the binding and control the binding equilibrium between subsites B_2R_2 and B_1R_1. (3) There is no overlapping between the binding sites occupied by the labeling nucleotide in derivative II ($B_3R_3p_2$) and the primary binding site for purine mononucleotides ($B_2R_2p_1$).

RNase A[1] hydrolyzes RNA in two steps: In the first one, the transesterification step, a cyclic phosphate is formed. This cyclic nucleotide is then hydrolyzed in the second step to the corresponding 3′-mononucleotide. The second step takes place only if the base of the cyclic nucleotide to be cleaved is a pyrimidine. However, the first step can be carried out, but at a much slower rate, even if the base in the 3′ position is a purine (Richards & Wyckoff, 1971; Cozzone & Jardetzky, 1977). The rate of hydrolysis in the first step is faster when a purine nucleoside is attached to the 5′ position, reaching its highest value with adenosine (Witzel & Barnard, 1962). Free adenosine also increases the rate of the second step of the catalysis (Wieker & Witzel, 1967). The studies carried out to clarify the mechanism by which this enhancement is produced have given general information about the binding subsites of RNase A. X-ray diffraction studies (Richards &

† From the Departament de Bioquímica, Facultat de Ciències and Institut de Biologia Fonamental, Universitat Autònoma de Barcelona, Bellaterra, Spain (C.A., R.L., and C.M.C.), and the Istituto Chimico, Università di Napoli, Napoli, Italy (L.P. and R.N.). *Received December 16, 1981; revised manuscript received May 18, 1982.*

[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; Ado, adenosine; Guo, guanosine; N^1-oxoAdo, N^1-oxoadenosine; cl^{6}PurRib, 9-β-D-ribofuranosyl-6-chloropurine; cl^{6}RMP, 9-β-D-ribofuranosyl-6-chloropurine 5′-phosphate; br^{8}Ado, 8-bromoadenosine; Up[CH_2]A, the phosphonate analogue of UpA in which the oxygen atom of the phosphoester bond between the phosphate and the adenosine moiety is replaced by a $-CH_2$ group; C2′–5′A, cytidylyl(2′–5′)adenosine; CM, carboxymethyl; NMR, nuclear magnetic resonance. The nomenclature of the RNase A binding subsites is found in Parés et al. (1980). B stands for base, R for ribose, and p for phosphate. $B_1R_1p_1$ is the main 3′-pyrimidine nucleotide binding site and $B_2R_2p_1$ the main 5′-purine nucleotide binding site. p_0 and p_2 are adjacent phosphate-binding sites.

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