INTERACTION OF SALICYLATE AT THE AMP SITE OF FRUCTOSE 1,6-BISPHOSPHATASE

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1. Introduction

As a result of X-ray crystallographic studies, it is becoming evident that similar structural domains for the binding of mononucleotide units are present in a variety of enzymes. A basic mononucleotide structural unit is present twice in the NAD-binding domain of dehydrogenases, and is also present in several kinases (for reviews, see [1] and [2]). The adenosine moiety of the coenzyme is bound in a hydrophobic pocket at one end of the binding crevice, which appears to be a general hydrophobic cleft for the binding of an aromatic molecule. As stated by Blake and Evans [3], the possibility of an evolutionary relationship in the nucleotide binding units of dehydrogenases and kinases further suggests that other classes of enzyme may also contain this basic unit. Indirect evidence, based on the interaction of several proteins with blue dextran–Sepharose columns [4,5], has also led to the prediction that structural features resembling the NAD-binding domain are present in many enzymes of yet unknown three-dimensional structure. Among those enzymes mentioned by Stellwagen and coworkers [4,5] are two enzymes, such as phosphorylase *a* and Fru-P_2ase, which bind a mononucleotide (AMP) at an allosteric site.

Einarsson et al. [6] have elegantly demonstrated by X-ray crystallography that salicylate, a general coenzyme competitive inhibitor of dehydrogenases [7], binds in the hydrophobic adenosine-binding pocket of alcohol dehydrogenase in a manner entirely similar to adenosine, in spite of their very different structures. The above finding led them to suggest that salicylate may act as a specific inhibitor of additional enzymes having similar binding domains. Therefore, if the allosteric site of Fru-P_2ase has structural features similar to the adenosine-binding domain of dehydrogenases, one should be able to demonstrate that salicylate interacts at the allosteric AMP site of Fru-P_2ase. The present work provides evidence of this interaction.

Abbreviations: Fru-P_2ase, fructose 1,6-bisphosphatase; NBS_2, 5,5′-dithiobis (2-nitrobenzoic acid).

2. Materials and methods

Pig kidney Fru-P_2ase with optimal activity at neutral pH was purified as previously described [8], with an additional final step involving heating 5 min at 62°C. The purified enzyme showed a single protein band (mol. wt 35 000) in polyacrylamide disc-gel electrophoresis in the presence of sodium dodecyl sulfate. Its protein concentration was determined by its absorbancy at 280 nm using the extinction coefficient $E^{1\%}_{1cm}$ of 7.55 [9]. The molecular weight of the enzyme tetramer was taken as 140 000 for all calculations. Fru-P_2ase activity was measured spectrophotometrically as described [10]. The assays were carried out at 30°C in a reaction system which contained 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes)–NaOH buffer (pH 7.5), 0.17 mM fructose 1,6-bisphosphate (Sigma stock 750-1), 5 mM $MgSO_4$, 0.1 mM EDTA, 0.3 mM NADP, phosphoglucose isomerase (2.3 U/ml), glucose-6-P dehydrogenase (0.6 U/ml), and other additions as indicated. The reaction was initiated by the addition of fructose 1,6-bisphosphate.

Chemical modification reactions were performed under the conditions given in the figure and table legends. 5-Iodosalicylic acid (Aldrich) was recrystallized several times from 25% (v/v) acetone–water. Salicylic

(Eastman Kodak) and iodosalicylic acid solutions were neutralized with NaOH.

3. Results and discussion

Experiments on the effect of salicylate on Fru-P_2ase activity showed that salicylate (up to 20 mM) had no effect on the activity of the enzyme. However, the presence of salicylate resulted in a significant relief of allosteric AMP inhibition (fig.1). Iodo-substituted salicylate proved to be more effective in diminishing AMP inhibition of Fru-P_2ase. The effect of 2 mM 5-iodosalicylate on AMP inhibition (fig.1, filled circles) was similar to that obtained with 10 mM salicylate. These results are entirely consistent with the binding data of salicylates in the adenosine-binding pocket of liver alcohol dehydrogenase [6], and suggest that salicylates also interact at the AMP site of Fru-P_2ase.

To test further whether AMP and salicylate bind at the same site on the enzyme, chemical modification experiments were performed. Previous reports have already demonstrated that, under certain experimental conditions, the AMP inhibition of Fru-P_2ase can be decreased or nearly abolished by means of chemical modification of the enzyme with pyridoxal-P [9,11, 12]. The loss of AMP inhibition can be prevented by the presence of AMP in the modification reaction system. Thus, if salicylate binds at the AMP site, it should also protect from loss of AMP inhibition. As shown in table 1, iodosalicylate afforded considerable

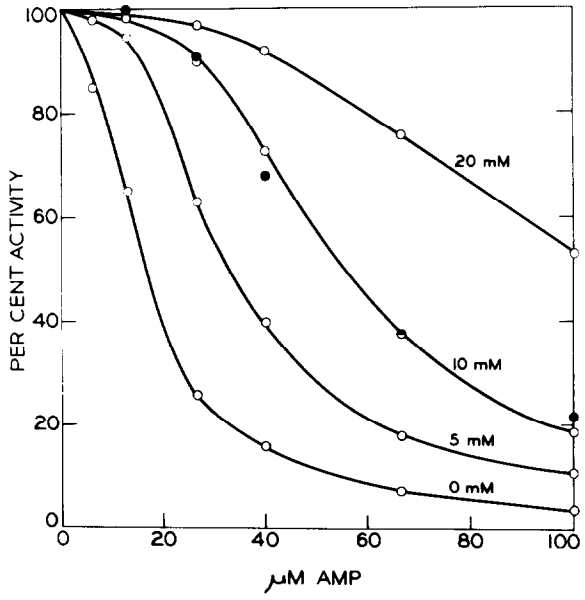

Fig.1. Effect of salicylate on the inhibition of fructose 1,6-bisphosphate by AMP. Enzyme activity was determined as described under Materials and methods, except for the variable AMP concentration (as indicated in the abscissa), and sodium salicylate (0–20 mM) as shown. The results indicated by *filled* circles (•) correspond to the activity of the enzyme measured in the presence of 2 mM iodosalicylate, and AMP as indicated in the abscissa.

Table 1
Effect of iodosalicylate on modification of fructose 1,6-bisphosphatase by pyridoxal-*P*

Enzyme treatment	Specific activity	Percent inhibition by 67 μM AMP	Pyridoxyl-P incorporated (mol/mol)
1. None	13.3	93	–
2. $NaBH_4$ control	12.8	91	–
3. Modified	10.8	24	3.5
4. Modified in the presence of 10 mM iodosalicylate	10.4	71	2.6
5. Modified in the presence of 0.5 mM AMP	12.3	93	2.2

Modification of Fru-P_2ase with pyridoxal-P was performed in a reaction system containing 100 mM sodium borate buffer (pH 8.0), 165 mM KCl, 66 μM EDTA, 1 mM fructose 1,6-bisphosphate, 8 mM pyridoxal-P, 7.3 μM Fru-P_2ase and other additions as indicated. Incubation was at 4°C, and after 18 min, the reaction was stopped with $NaBH_4$ (for additional details, see [12]). The control enzyme (exp. 2) was subjected to the same treatments, except for the omission of pyridoxal-P

protection against loss of AMP inhibition by pyridoxal-P treatment and measurements of the incorporation of pyridoxal-P into the protein demonstrate that iodosalicylate (as does AMP) also decreases the incorporation of reagent into Fru-P_2ase.

If salicylate binds at the AMP site, as it appears to do from the above results, it still remains to be explained why it does not act as an inhibitor of Fru-P_2ase. A likely explanation can be suggested if one considers that the inhibition of an enzyme by an allosteric inhibitor involves (in its simplest form) at least two steps: (a) binding of the inhibitor at the allosteric site; followed by (b) a conformational change of the protein which results in an unfavorable alignment of either substrate binding or catalytic groups at the active site. Thus, in this simple scheme, salicylate could bind at the allosteric site but might not induce the conformational change that results in inhibition of enzyme activity. To test the above possibility, advantage has been taken of an unexplored feature of the titration of SH groups of Fru-P_2ase with NBS_2. Previous reports on the titration of various Fru-P_2ases with an excess NBS_2 (see [13] and references cited therein) have shown the very fast reaction of 4 SH groups per molecule (i.e., 1 subunit) of enzyme, followed by (depending on the reaction conditions) a much slower reaction of further SH groups. However, no attempt has been made to limit the reaction to only the fast reacting SH groups by using stoichiometric amounts of NBS_2. Using this approach, it was found that the reactivity of the fast reacting SH (fig.2A, empty circles) is dramatically increased by the presence of AMP (fig.2A, empty squares), which is most likely the reflection of an AMP-induced enzyme conformational change. Conversely, iodosalicylate does not alter the SH reactivity (fig.2A, filled circles), in accordance with its inability to inhibit the activity of the enzyme.

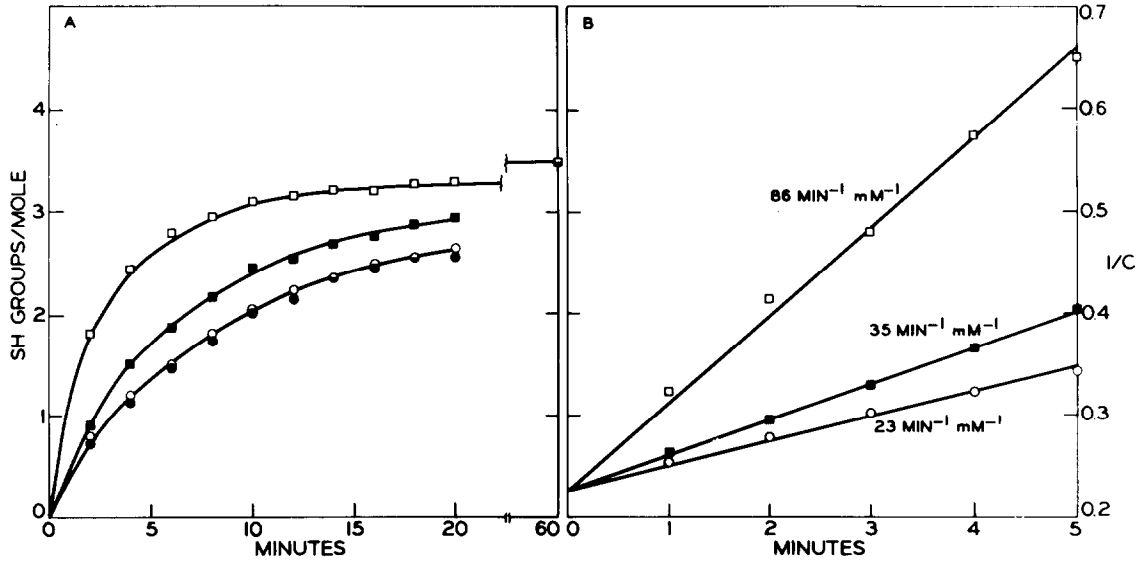

Fig.2A. Reaction of fructose 1,6-bisphosphatase with NBS_2. Enzyme (1.1 μM) was allowed to react at 28°C with 4.4 μM NBS_2 in a reaction system containing 60 mM Tris–HCl buffer (pH 7.5), 25 mM KCl, 120 μM EDTA, and other additions as indicated below. (○), No other additions; (□), plus 33 μM AMP; (●), plus 2 mM iodosalicylate; (■), plus 33 μM AMP and 2 mM iodosalicylate. Titration of enzyme SH groups was followed by the rate of formation of the thionitrobenzoate anion at 412 nm with a Gilford Model 2000 spectrophotometer. Reactions were always started by the addition of NBS_2. A molar extinction coefficient of 13 600 for the liberated thionitrobenzoate anion was used for all calculations [14]. Fig.2B. Second-order rate constants for the titration of the SH group with NBS_2. Data obtained from fig.2A were plotted as $1/C$ against time, applicable for this special case in which the concentrations of A (NBS_2) and B (reactive SH) at zero time are identical. The rate law is then given by the equation $1/C - 1/C_0 = kt$, in which C is the remaining concentration of A or B at any time [15]. Second-order rate constants (k) are given in the figure.

However, iodosalicylate competes with AMP since in the presence of both (fig.2A, filled squares) the SH reactivity is intermediate to that found with and without AMP. The second-order rate constants for the titration of the SH group with NBS_2 are given in fig.2B. Experiments with salicylate, instead of iodosalicylate, gave essentially the same type of result. The rate constant for the titration of the SH group was 20 min^{-1} mM^{-1} in the presence of 8 mM salicylate, and 33 min^{-1} mM^{-1} in the presence of 8 mM salicylate plus 33 μM AMP.

In conclusion, the results presented herein provide evidence for the interaction of salicylate with Fru-P_2ase and that this interaction most likely occurs at the AMP site. Based on previous knowledge of the interaction of salicylate with alcohol dehydrogenase [6], and related evidence (for details, see refs. [1–5]), it may be tentatively suggested that the allosteric site of fructose 1,6-bisphosphatase has structural features similar to the adenosine-binding domain of dehydrogenases and kinases.

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