Biochemical Pharmacology, Vol. 29, pp. 2113–2116.

0006-2952/80/0801-2113 $02.00/0

SALICYLATE INHIBITION OF MONKEY MUSCLE CREATINE KINASE

W. RICHARD CHEGWIDDEN* and DAVID C. WATTS
Biochemistry Department, Guy's Hospital Medical School, London SE1 9RT, U.K.

(*Received* 24 *October* 1979; *accepted* 31 *March* 1980)

Abstract— Creatine kinase is inhibited in both the forward and back reactions by salicylate compounds. The inhibition process is complex, resulting in curved Dixon plots, and is non-competitive with respect to creatine. Salicylate does not affect the inhibition caused by chloride ions but reduces the activation by acetate. The ionizations responsible for the pH/activity curve are also suppressed. These results are interpreted to indicate that more than one salicylate molecule binds per active centre at a site away from that for the transferable phosphoryl group. Inhibition probably involves binding to a particular enzyme conformational state that also prevents activation by acetate.

The inhibition of rabbit muscle creatine kinase (ATP: creatine phosphotransferase, E.C.2.7.3.2) by simple anions has been known for a long time but only recently has it been realized that they may be classified in terms of the kinetic nature of the effect and the specific nature of the binding site [1]. In this way three classes of anions were recognized. Class I anions, such as acetate, activate the enzyme in the direction of ATP synthesis. Class II anions, such as NO_3^- and Cl^-, appear to mimic the transferable phosphoryl group and stabilized the formation of the dead end complex, enzyme–creatine–MgADP. Class III anions, such as SO_4^{2-}, are competitive inhibitors of MgATP and phosphocreatine but noncompetitive inhibitors of creatine and MgADP and presumably bind at the site for the transferable phosphoryl group in its normal tetrahedral configuration.

Much of this work has been confirmed and extended by using the creatine kinase isolated from rhesus monkey skeletal muscle [2, 3]. More recently our attention has turned to the investigation of pharmacologically active anions. The present results report on the complex properties of some salicylate compounds that appear to act in a manner different from the three classes so far recognized.

MATERIALS AND METHODS

Creatine kinase was purified from the skeletal muscles of *Macaca mulatta* as described previously [2]. The preparations had specific activities in the range 130–170 μ equivalent H^+/mg protein as determined by the pH-stat assay at pH 8.6 in unbuffered solution at 30° for the forward reaction [2] (phosphocreatine synthesis). The back reaction was also assayed titrimetrically but at pH 8.0 [2]. The compositions of the reaction mixtures used are given in the legends to the figures and tables.

All reagents used were as described previously [2] or were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

RESULTS

Table 1 compares sodium salicylate with a series of related compounds as inhibitors of monkey muscle creatine kinase. Enzyme activity is measured in the direction of phosphocreatine synthesis (forward direction). This inhibition is reversed by dialysis or dilution. The effects of sodium sulphate and sodium lactate are also given for comparison. In these experiments it is not possible to control the ionic strength but the lack of inhibition by lactate shows that any non-specific ionic strength effect is negligible. Inhibition by the salicylate series is noticably greater than with benzene sulphonate, although the introduction of additional or larger substituents into the aromatic ring decreases effectiveness. Benzene sulphonate is itself one-third more potent as an inhibitor than the plain sulphate anion.

Salicylate was also found to inhibit creatine kinase in the direction of ATP synthesis, although to a lesser extent than in the forward direction. The effects on the forward reaction of varying the concentrations of the salicylate compounds are shown in Fig. 1. The data are expressed as Dixon plots [4] and, instead of the usual linear form, give curves which are concave upwards for all three inhibitors. This suggests there is binding of more than one inhibitor molecule to the same site on the enzyme or to the same kinetic form. Alternatively, the binding of the first molecule is promoting the binding of subsequent molecules in a cooperative manner. Since all the salicylate compounds behave in much the same way, subsequent investigations were carried out only with sodium salicylate which, because of its greater solubility, was most easy to handle.

Figures 2 (a and b) show, also as Dixon plots, the effect of varying the concentration of sodium salicylate with two lower concentrations each of creatine and MgATP than those used for Fig. 1. It can be

* Present address: Department of Biological Sciences, Sheffield City Polytechnic, Pond Street, Sheffield S1 1WB, U.K.

Table 1. Inhibition of creatine kinase activity in the forward reaction by various anions*

Source of anion	Concentration (moles/l)	Inhibition (%)
Sodium salicylate	0.10	93
Sodium salicylate	0.05	70
Sodium acetyl salicylate	0.05	50
Sodium *p*-amino salicylate	0.05	38
Sodium benzene sulphonate	0.10	43
Sodium sulphate	0.10	33
Sodium lactate	0.10	0
Sodium glycollate	0.10	0
Sodium pyruvate	0.10	0

* The reaction mixture comprised creatine, 40 mmoles/l; ATP, 4 mmoles/l; magnesium acetate, 5 mmoles/l and mercaptoacetic acid, 1 mmole/l.

seen that the plots are still of the same curved form showing that the departure from linearity does not depend on substrate concentration. The same data expressed as double-reciprocal plots (not shown) suggested, assuming these to be linear, that inhibition may be non-competitive with respect to creatine but competitive with respect to MgATP.

When the effect of varying the salicylate concentration was tested with the back reaction a similar plot to that obtained in Fig. 1 was obtained, showing that the same complexity of binding also applies in the direction of ATP synthesis.

As mentioned in the introduction, chloride ions are believed to inhibit creatine kinase by binding at the site of the transferable phosphoryl group [1].

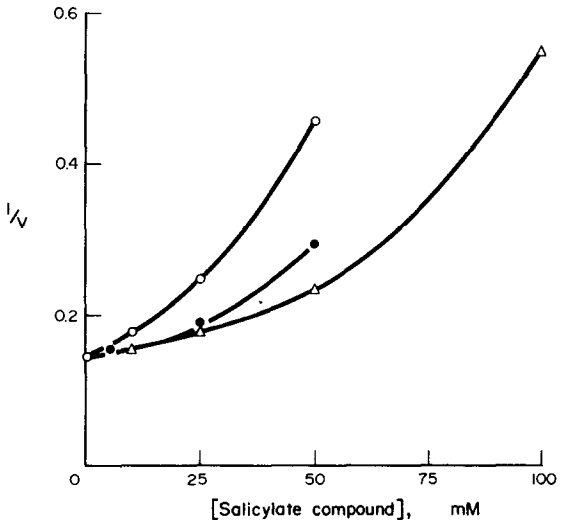

Fig. 1. A comparison of the effects of varying the concentration of sodium salicylate, sodium acetyl salicylate and sodium *p*-amino salicylate on the reciprocal of the relative initial velocity (v) of the creatine kinase forward reaction. (○) Plus sodium salicylate; (●) plus sodium acetyl salicylate; (△) plus sodium *p*-amino salicylate. Concentrations of other components of the reaction mixture were: creatine, 40 mmoles/l; ATP, 4 mmoles/l; magnesium acetate, 5 mmoles/l; mercaptoacetic acid, 1 mmole/l. The enzyme concentration was 1.3 $\mu g/cm^3$.

Figure 3 shows that this inhibition by chloride ions is not affected by progressively increasing the concentration of sodium salicylate. Hence it may be concluded that salicylate binding does not involve interaction with the transition state or a quasi-transition state of the enzyme.

In order to examine further the nature of salicylate inhibition the concentration of salicylate was varied

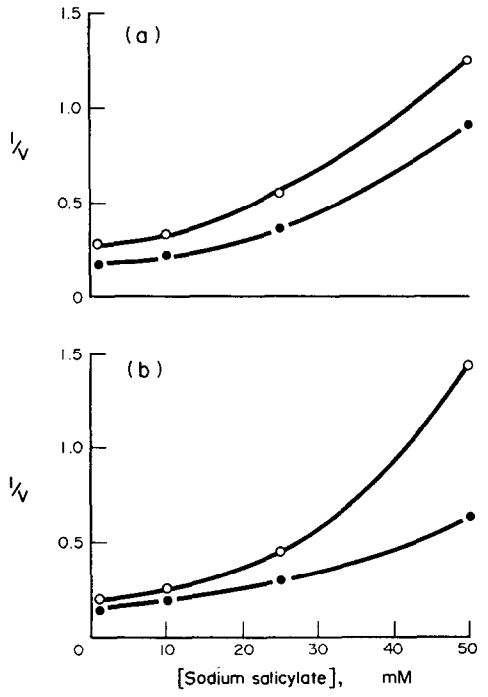

Fig. 2. Panel a: Dixon plots obtained for the creatine kinase forward reaction by varying the concentration of sodium salicylate at fixed creatine concentrations. (○) Plus 10 mmoles/l creatine; (●) plus 20 mmoles/l creatine. Panel b: Dixon plots obtained for the creatine kinase forward reaction by varying the concentration of sodium salicylate at fixed MgATP concentrations. (○) Plus 1 mmole/l ATP; (●) plus 2 mmoles/l ATP. The magnesium acetate concentration was fixed at 1 mmole/l above the ATP concentration in each case. Concentrations of reaction mixture components were as in Fig. 1 except where otherwise stated.

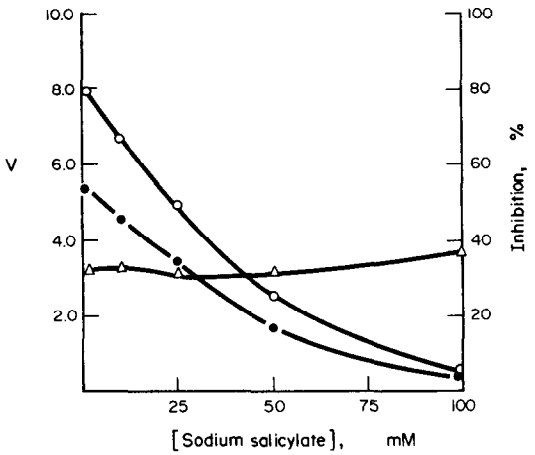

Fig. 3. Inhibition of the creatine kinase forward reaction by sodium chloride in the presence of varying sodium salicylate concentrations. (○) Relative initial velocity in the absence of sodium chloride (V_1); (●) relative initial velocity in the presence of sodium chloride, 100 mmoles/l (V_2); (△) per cent inhibition by 100 mmoles/l sodium chloride

i.e. $\frac{(V_1 - V_2)}{V_1} \times 100\%$. Other conditions were as in Fig. 1.

in the presence and absence of acetate. Figure 4 shows that acetate activates in the forward direction of the creatine kinase reaction. As the salicylate concentration is increased this activation is progressively abolished. From these results it may be inferred that there is competition between the two anions although they need not necessarily bind at the same site on the enzyme. Figure 5 shows the

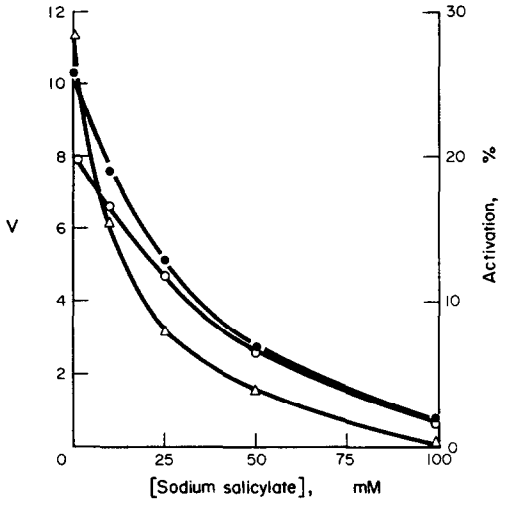

Fig. 4. Activation of the creatine kinase forward reaction by sodium acetate in the presence of varying sodium salicylate concentrations. (○) Relative initial velocity in the absence of sodium acetate (V_1); (●) relative initial velocity in the presence of sodium acetate, 100 mmoles/l (V_2); (△) per cent activation by 100 mmoles/l sodium acetate

i.e. $\frac{(V_2 - V_1)}{V_1} \times 100\%$. Other conditions were as in Fig. 1.

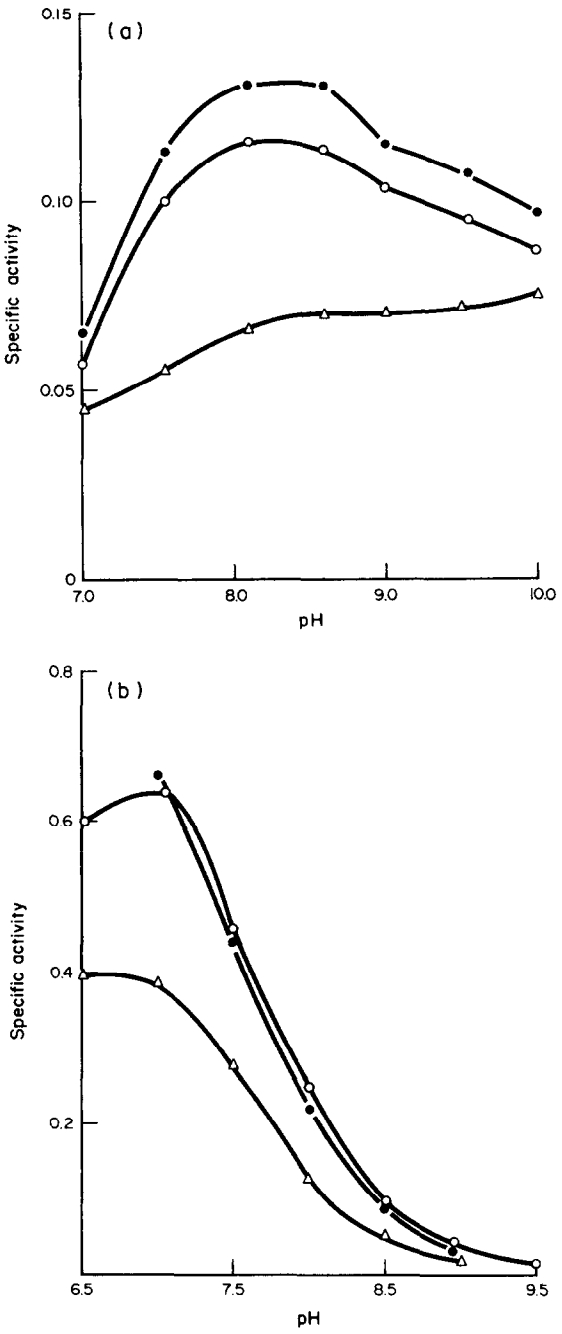

Fig. 5. The effect of pH on creatine kinase activity in the forward reaction (panel a) and the back reaction (panel b) (○) with no added anions; (●) in the presence of 0.1 mmole/l sodium acetate; (△) in the presence of 0.025 mole/l sodium salicylate. Concentrations of other assay components were as described in Table 1 for the forward reaction; for the back reaction these were 10 mmole/l phosphocreatine, 1.25 mmole/l ADP, 2 mmole/l magnesium acetate and 1 mmole/l mercaptoacetate. The specific activity is expressed as μmoles H^+ released or absorbed/min/μg protein.

effect of pH on the inhibition of the forward and back reactions by a fixed concentration of sodium salicylate. Substrate conditions for the forward reaction give approximately 75 per cent of V_{max}. It can be seen (Fig. 5a) that the presence of salicylate

effectively suppresses the normal pH curve. Similar, though not so striking results are obtained for the back reaction (Fig. 5b).

DISCUSSION

Although the concentrations of salicylate compounds required to inhibit creatine kinase are considerably greater than those required for pharmacological effects, they are considerably more potent than simpler anions such as sulphate, chloride or lactate. Furthermore, the inhibition, although complex, appears to be fairly specific and all three salicylate compounds appear to behave in much the same way with the binding of more than one molecule being involved in the inhibition. This applies to both the forward and back reactions catalysed by the enzyme. As with the simpler anions, inhibition is reversed by dialysis or dilution (Fig. 1). A similar pattern of inhibition has been reported for the inhibition of plasma amine oxidase by the antidepressant drug, clorgyline [5]. Unlike that system, however, a semi-log dose–response curve for the inhibition of creatine kinase by salicylate showed no indication of a biphasic reaction.

The salicylate binding site seems to be distinct from the binding site for chloride ions (Fig. 3) so that inhibition by this drug is unlikely to result from direct interaction at the site of the transferable phosphoryl group. There is clearly an interaction with the acetate binding site (Fig. 4) providing evidence that the acetate and chloride binding sites are distinct from each other. However, while acetate activates competitively with respect to creatine [3], salicylate inhibits non-competitively with respect to creatine. Since activation by acetate is most probably mediated by inducing a specific conformational change in the enzyme [3] it seems reasonable to conclude that salicylate inhibits by binding to a different conformational form of the enzyme.

The effect of salicylate on the pH profiles of creatine kinase suggests that inhibition also involves an effect on the normal ionizations that are responsible for the bell-shaped activity curve. Thus, in the forward direction the percentage inhibition by salicylate increases with pH as the enzyme activity increases but falls off again as the enzyme activity falls. This results in the pH profile in the presence of salicylate becoming almost flat (Fig. 5a).

Salicylate was found to give a qualitatively similar pattern of inhibition for the back reaction (ATP synthesis) as for the forward reaction. Under the conditions used the extent of inhibition was less pronounced, as illustrated by the effect on pH (Fig. 5b). This may be partly because while there appears to be a negligible ionic strength effect on the forward reaction (Table 1), there is a marked ionic strength effect on the back reaction caused by decreasing the stability constant for MgADP [6]. As free ADP is itself inhibitory, investigations of other modifiers of the back reaction, such as salicylate, should be interpreted with extreme caution. It was shown by Mahowald *et al.* [7] that rabbit muscle creatine kinase was inhibited extremely rapidly by 2,4-dinitrofluorobenzene and that this occurred by reaction with the reactive thiol group on the enzyme. It seems possible from the general similarity in structure that this reagent could act by binding to the same site on the enzyme as is used by salicylate. The effect of salicylate compounds on the reactive thiol group and on its inhibition by 2,4-dinitrofluorobenzene would make an interesting future study.

Acknowledgements—A grant from the Muscular Dystrophy Group of Great Britain in support of this research and the personal support of W. R. C. is gratefully acknowledged.

REFERENCES

1. E. J. Milner-White and D. C. Watts, *Biochem. J.* **122**, 727 (1971).
2. W. R. Chegwidden and D. C. Watts, *Biochim. biophys. Acta* **410**, 99 (1975).
3. W. R. Chegwidden, Ph.D. Thesis, University of London (1974).
4. M. Dixon, *Biochem. J.* **55**, 170 (1953).
5. M. D. Houslay and K. F. Tipton, *Biochem. Pharmac.* **24**, 429 (1975).
6. R. C. Phillips, S. J. Philip George and R. J. Rutman, *J. Am. chem. Soc.* **88**, 2631 (1966).
7. T. A. Mahowald, E. A. Noltmann and S. A. Kuby, *J. biol. Chem.* **237**, 1535 (1962).