EFFECTS OF HIGH CONCENTRATIONS OF SALT ON THE ESTERASE ACTIVITY OF HUMAN CARBONIC ANHYDRASE

H. STEINER and S. LINDSKOG

Department of Biochemistry, University of Göteborg and Chalmers Institute of Technology, Fack, S-402 20 Göteborg 5, Sweden

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1. Introduction

Most monovalent anions inhibit the zinc-containing enzyme carbonic anhydrase [1]. It has been shown that these anions bind to the active site at a position close to the metal ion [2, 3], and it has been proposed that the inhibiting anion displaces a metal-coordinated OH^- involved in the catalytic reaction [4, 5]. Divalent anions do not seem to inhibit the enzyme [6], and buffer solutions containing SO_4^{2-} as the anionic component have been employed in most recent kinetic studies of carbonic anhydrase [1, 7].

During the course of an investigation of human carbonic anhydrase C, aiming at correlating properties of the enzyme in solution with the recently determined crystal structure [8], we found that high concentrations of salts containing polyvalent anions, such as are used for the crystallization of the enzyme, cause a pronounced activation of the enzyme-catalyzed hydrolysis of *p*-nitrophenyl acetate. The results presented in this paper suggest that the increase of the esterase activity mainly results from the effect of ionic strength on the chemical potential of the substrate in aqueous solution.

2. Materials and methods

The two major forms of human carbonic anhydrase, B and C, were prepared by a method similar to that described by Armstrong et al. [9], but the final purification was achieved by chromatography on DEAE-cellulose (Whatman DE-23). Reagent grade chemicals were used without further purification. Protein concentrations were estimated spectrophotometrically at 280 nm taking molar absorbancies of 48,900 $M^{-1}cm^{-1}$ and 56,000 $M^{-1}cm^{-1}$ for the B and C forms, respectively.

Conditions for the assay of *p*-nitrophenyl acetate hydrolysis have been described previously [10]. The release of product was followed at the isosbestic wavelength for *p*-nitrophenol and *p*-nitrophenolate anion (348 nm). Possible effects of salt on the isosbestic wavelength were neglected. A control experiment in 2 M $MgSO_4$ indicated a shift of 2 nm or less. The increment in molar absorbancy at 348 nm for the reaction was taken as 5,150 $M^{-1}cm^{-1}$ [11]. The addition of 1 M salts changed this value 5% or less, and no correction was applied, except in case of $MgSO_4$ where concentrations up to 2 M were employed.

Because *p*-nitrophenyl acetate is slowly hydrolyzed in water or neutral buffers, a rapid method for solubility measurements was required. We employed a type of Brönsted saturator [12]; a 10 cm long glass column having an inner diameter of 1 cm was packed with a 3 cm layer of *p*-nitrophenyl acetate crystals. The column was supplied with a water jacket for temperature control. After the introduction of salt solution or buffer on top of the ester layer, an aliquot of 0.5 M *p*-nitrophenyl acetate in acetone was injected into the solution to give a final acetone concentration of 1% (v/v). The amount of ester was chosen to be sufficient for supersaturation, and a precipitate of ester was always formed. The solution was then passed through the column at a flow rate of 1.5 ml/min by means of a peristaltic pump. The concentrations of ester in the saturated solutions thus obtained were usually estimated at 400

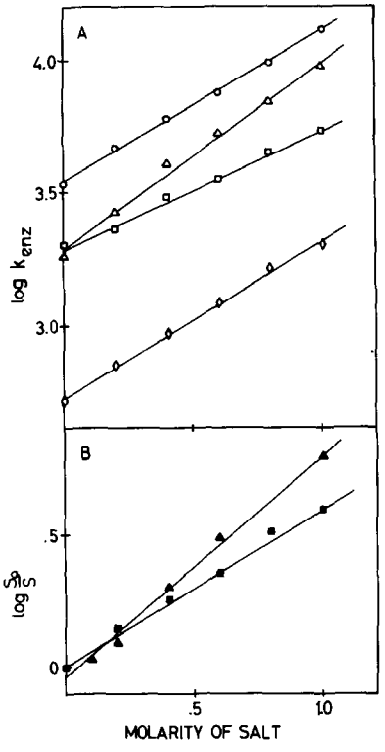

Fig. 1. A) Effect of salt concentration on log k_{enz}. (□-□-□): Na_2SO_4 and human C enzyme, pH 7.5; (○-○-○): Na_2SO_4 and human C enzyme, pH 9.0; (△-△-△): Na_3-citrate and human C enzyme, pH 7.5; (◇-◇-◇): Na_3-citrate and human B enzyme, pH 7.5. Temperature, 25°. Buffers: 0.05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate, pH 7.5, or 0.05 M Tris sulfate, pH 9.0. Substrate concentration, 0.4 mM. Acetone concentration, 1% (v/v). Enzyme concentrations, human C enzyme, 2.0 μM; human B enzyme, 7.8 μM. B) Effect of salt concentration on $\log(s_0/s)$, where s_0 and s are the solubilities of *p*-nitrophenyl acetate in the absence and presence of salt, respectively. (■-■-■): Na_2SO_4; (▲-▲-▲): Na_3-citrate. Temperature, 25°. Acetone concentration, 1% (v/v).

nm after dilution and hydrolysis in 0.1 M NaOH, taking $\epsilon_{400} = 18{,}100\ M^{-1}cm^{-1}$ for the *p*-nitrophenolate anion [11]. In case of $MgSO_4$, ester concentrations were estimated directly at 310 nm and corrections for the effect of salt on the molar absorbancy were applied.

3. Results

The effects of sodium sulfate and trisodium citrate on the esterase activity of the human carbonic anhydrases B and C are shown in fig. 1A. As the enzyme initial rates, v_0, are proportional to the substrate concentration in the whole attainable range (see [11]), the activities are presented as the apparent second order rate constants, k_{enz}, defined by the relation $v_0 = k_{enz}\,[E]\cdot[S]$.

The decrease of the solubility of *p*-nitrophenyl acetate in solutions of the same salts is illustrated in fig. 1B. The slopes of the straight lines obtained in these logarithmic plots are given in table 1 together with some additional data on Na_2SO_4 at 4.5° and on $MgSO_4$. Enzymic rate enhancements of similar magnitudes were also observed with $(NH_4)_2SO_4$ and Na_2HPO_4.

The effects of 1 M Na_2SO_4 on the pH dependence of the esterase activity of human carbonic anhydrase C are shown in fig. 2.

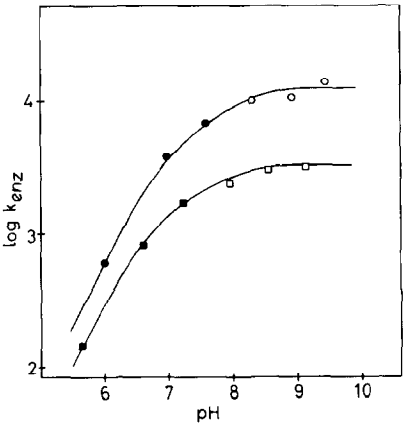

Fig.2. The pH dependence of log k_{enz} for the human C enzyme in the presence and absence of 1 M Na_2SO_4, respectively. Circles indicate the presence of salt and squares indicate no added salt. Closed symbols represent 0.05 M 2,2-bis(hydroxymethyl)-2,2',2''-nitrilotriethanol sulfate buffers; open symbols represent 0.05 M Tris sulfate buffers. Other conditions as in fig.1.

4. Discussion

The plots of log k_{enz} versus salt concentration are practically linear as shown in fig. 1 A. This finding led us to investigate whether the activation could be correlated with the effect of salt on the solubility of the substrate, since it is known that solubilities of non-

Table 1
Slopes of log k_{enz} versus salt concentration and the salting-out constants, K, for *p*-nitrophenyl acetate

Salt	Enzyme form	Temperature	pH	Slope of log k_{enz} versus salt molarity	Salting-out constant, K
Na_2SO_4	C	4.5	7.5	0.39	0.60
	C	25	7.5	0.44	0.60
	C	25	9.0	0.56	
$MgSO_4$	C	25	7.5	0.46	0.50
Na_3-citrate	C	25	7.5	0.70	0.83
	B	25	7.5	0.59	

The activity measurements were made in 0.05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate buffers, pH 7.5, or in 0.05 M Tris sulfate buffers, pH 9.0, containing various concentrations of salt.

electrolytes generally follow the empirical relation

$$\log(s_0/s) = K \cdot c,$$

where s and s_0 are the solubilities with and without added salt, respectively, K is a salting-out constant, and c is the molarity of added salt. The relative activity coefficient of the substrate, γ_S, is equal to s_0/s (see [13]).

As seen from the data in table 1, the plots of log k_{enz} versus salt concentration have slopes nearly equal to or somewhat less than the corresponding salting-out constants for the substrate with all studied salts and at 25° as well as at 4.5°. The correlation with the salting-out constants becomes closer if the pH dependence of the activation is taken into consideration (fig. 2). In 1 M Na_2SO_4 the apparent pK_a of the group controlling the activity is increased by approx. 0.3 units. This may be caused by a salt effect on the intrinsic pK_a of the ionizing group in the active site, or by a specific binding of an anion to the acidic form of this group in analogy to monovalent, inhibitory anions [1], or a combination of both effects. At pH 6 the activation by 1 M Na_2SO_4 is only 2-fold or 0.3 logarithmic units, while in the plateau region at pH 9 and above the activation is 0.6 units which corresponds closely with the observed salting-out constant for Na_2SO_4.

It would thus appear that the effects of high salt concentrations on the esterase activity of carbonic anhydrase can be described in quite simple terms. The major effect is a rate enhancement which is proportional to the relative activity coefficient of the substrate, γ_S. This activation is partly counteracted by a relatively small salt effect on the apparent ionization constant, K'_a, of the activity-linked group in the enzyme. Both effects can be summarized by the relation

$$k_{enz} = k^{\circ}_{enz}\ \gamma_S\ \frac{1}{1 + a_H/K'_a},$$

where k°_{enz} is independent of pH and ionic strength, K'_a depends on the ionic strength, and a_H is the activity of H^+ as measured by the glass electrode. Some experiments have been performed to see if the mild inhibition by organic solvents, which has been reported from several laboratories [11, 14, 15], can be similarly described. The results were complex, however, and must partly be explained as resulting from specific interactions with the protein (cf. [14]).

The molecular interpretation of the salt effects is less obvious, partly because the limited solubility of the ester restricts studies to substrate concentrations much smaller than K_m. Thus, $k_{enz} = k_{cat}/K_m$, and the addition of salt may affect either Michaelis–Menten parameter or both. In most cases effects of high salt concentrations on enzyme activities have been interpreted as resulting from effects on active site groups controlling substrate binding or catalytic steps, conformational changes, or effects on subunit association–dissociation equilibria (see [13]). Of course, substrate binding must also depend on the chemical potential of the substrate. The simple relation between k_{enz} and γ_S observed for carbonic anhydrase indicates that the major part of the activation might reflect an increase of the apparent affinity of the sub-

strate for the active site in proportion to the increase of γ_S at high salt concentrations, whereas the salt might have only a minor effect on the properties of the enzymic active site.

The enhancement of the esterase activity of carbonic anhydrase can be compared with the salt effects on the chymotrypsin-catalyzed hydrolysis of hippuric acid esters and the inhibition of chymotrypsin by aromatic hydrocarbons studied by Miles et al. [16]. They showed that the binding strengths of these substrates and inhibitors are increased at high salt concentrations, and this phenomenon could be interpreted as analogous to an extraction process, involving a "salting-out" of the ligand into the active site. Whether a similar model applies to carbonic anhydrase or not will require further studies with substrates giving measurable K_m values. Pocker and Watamori [17] recently reported relatively small K_m values for 3-acetoxy-2-nitropyridine. Unfortunately, we have not been able to reproduce their results, but find K_m unmeasurably large also for this substrate.

Acknowledgements

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