Biochimica et Biophysica Acta, 341 (1974) 345–356

EBA 67184

A CALORIMETRIC STUDY OF THE BINDING OF SULFONAMIDES AND CYANATE TO CARBONIC ANHYDRASE

JESSE S. BINFORD*, SVEN LINDSKOG** and INGEMAR WADSÖ
Thermochemistry Laboratory, Chemical Center, University of Lund, Lund (Sweden)
(Received November 22nd, 1973)

SUMMARY

The binding reactions between several sulfonamides and human carbonic anhydrase C and bovine carbonic anhydrase have been investigated by microcalorimetric methods. The binding of potassium cyanate to the bovine enzyme was also studied.

Measurements were performed in Tris and in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer at pH 8.20.

For the idealized process

$$(E\text{–}OH^- + NH_2SO_2R \rightleftharpoons E\text{–}\bar{N}HSO_2R + H_2O)aq$$

the following values were derived.

Human C enzyme, HEPES buffer: ΔH° (25 °C) = −45.7 kJ·moles^{-1} and −60.3 kJ·mole^{-1} for benzenesulfonamide and "CL 11.366", respectively. The corresponding ΔC_p values were found to be small, approx. +0.1 and −0.1 kJ·mole^{-1}·K^{-1}, respectively.

For the bovine enzyme the following ΔH° (25 °C) values were derived (kJ·mol^{-1}): benzenesulfonamide, −41.2 (HEPES) and −41.3 (Tris); *p*-toluenesulfonamide, −45.2 (Tris); sulfanilamide, −45.1 (Tris); methazolamide −59.0 (HEPES); "CL 11.366", −57.7 (HEPES).

For the binding of cyanate to the bovine enzyme both the equilibrium constant and the enthalpy change were determined (25 °C, HEPES buffer).

For the idealized reaction

$$(E\text{–}OH^- + OCN^- \rightleftharpoons E\text{–}OCN^- + OH^-)aq$$

the following values were derived: ΔG° = +12.2 kJ·mole^{-1}; ΔH° = +1.5 kJ·mole^{-1} and ΔS° = −36 J·mole^{-1}·K^{-1}.

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

* Present address: Department of Chemistry, University of South Florida, Tampa, Fl.
** Department of Biochemistry, University of Göteborg and Chalmers Institute of Technology, Sweden.

INTRODUCTION

The zinc-containing metalloenzyme carbonic anhydrase (carbonic hydro-lyase, EC 4.2.1.1) is specifically inhibited by monovalent anions and by aromatic sulfonamides. In particular, the interactions of the enzyme with sulfonamides have been intensely studied by a variety of techniques [1]. It has been shown by X-ray crystallography that the sulfonamide group binds directly to the metal ion while other parts of the inhibitor interact with groups in the active site cavity [2]. Presumably because of these multiple interactions sulfonamides are very strongly bound to the enzyme. Anions also bind close to the metal ion, but, at least in some cases, the metal ion–anion distance is appreciably greater than in simple model complexes [2].

To further characterize the thermodynamics of the formation of inhibitor–carbonic anhydrase complexes, we have determined the enthalpies for the binding of some inhibitors to the human C and the bovine enzymes.

MATERIALS AND METHODS

Preparations of solutions

Human carbonic anhydrase C and bovine carbonic anhydrase, form B, were prepared from erythrocytes by methods described previously [3, 4].

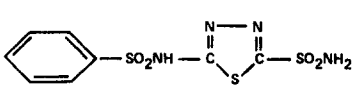
Stock solutions of the enzymes were stored at −20 °C. To prepare samples for the calorimetric experiments, concentrations were adjusted and the solutions were dialyzed against buffer at 4 °C. Buffer solutions were changed four times over a two- or three-day period. The enzyme solutions thus prepared were kept at 4 °C. Concentrations were determined with a Shimadzu QV-50 spectrophotometer at 280 nm assuming molar absorption coefficients of $5.6 \cdot 10^4$ M^{-1} cm^{-1} for the human and $5.7 \cdot 10^4$ M^{-1} cm^{-1} for the bovine enzyme [5] and a molecular weight of 30 000 for both enzymes [5].

Buffer solutions were prepared and adjusted to pH 8.20 using a Radiometer Model 26 pH meter as follows: 0.05 M Tris (Merck, p.a.) was prepared using H_2SO_4, and 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (Schwarz/Mann) was prepared using NaOH. Reagent grade acid and base were used and deionized glass-distilled water.

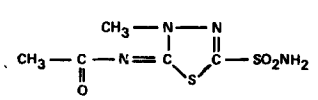
Benzenesulfonamide (Fluka) was recrystallized in water and ethanol. *p*-Toluenesulfonamide and sulfanilamide (Eastman) were used without further purification (gift from Dr F. Fölsch, Göteborg).

Methazolamide (5-acetylimino-4-methyl-D^2-1,3,4-thiadiazoline-2-sulfonamide and "CL 11.366" (5-benzenesulfonamido-1,3,4-thiadiazole-2-sulfonamide; cf. Fig. 1)

"CL 11.366"



METHAZOLAMIDE



were gifts from Dr P. Wistrand, Uppsala, and were used without further purification.

Potassium cyanate (BDH) was purified by repeated crystallization from water (45 °C) and was dried in vacuum.

The inhibitor solutions were prepared by weighing the solids and dissolving in portions of the same stock buffer used in dialysis of the enzymes.

Calorimetric measurements on human carbonic anhydrase C

All heat measurements on human carbonic anhydrase C were made in the LKB batch type microcalorimeter Model 10700-2, using gold vessels [6]. Frequent electrical calibrations were performed throughout the period of study.

A series of heats of dilution of sucrose were measured and found to be consistent with earlier work [6]. In all runs on heats of reaction, 4 ml enzyme solution and 2 ml inhibitor solution, measured by weighed syringe, were placed on the reaction side and equal amounts of buffer were placed on the reference side. Careful cleaning of reaction vessels was necessary between runs, first with dilute NaOH, then dilute HCl followed by distilled water and finally buffer. The buffer solution was carefully aspirated out, but the vessels were not dried. Heats of dilution were determined by using 4 ml enzyme and 2 ml buffer on the reaction side with buffer only on the reference side. Thermal equilibrium was usually attained within two hours after filling and closing the calorimeter. The signal produced on mixing was amplified by a Keithley Model 148 nanovoltmeter and recorded on a Sargent SR recording potentiometer fitted with a ball and disc integrator. After the base line was regained, which usually required about 30 min, a background correction was made by repeating the mixing cycle two or three times.

The temperature of the reaction vessels was measured with a calibrated thermistor enclosed within the heat sink and equilibrated overnight. The estimated accuracy of this temperature measurement was $\pm$ 0.02 °C.

Calorimetric measurements on bovine carbonic anhydrase

Measurements on bovine carbonic anhydrase were made in a prototype of the LKB Model 10700-1 flow microcalorimeter [7]. The signal was amplified by a Keithley Model 150 B microvolt ammeter which was connected to a Servogor Model RE 511 recording potentiometer. Screw-driven syringe pumps, using Hamilton 10-ml syringes, were used to control the flow of the reactants. Flow rates for each gear setting were measured by timed flow in a calibrated 1-ml pipet. The standard deviation of every flow rate used was less than 0.2%. The calorimeter was calibrated electrically while flowing water over a range of flow rates and the calibration constant for the appropriate flow rate was used in calculating the reaction heats. During a heat of reaction measurement approx. 10 min flow was allowed to reach a steady-state heat output rate. The contact time in the reaction section of the tubing was 2 min at the fastest flow rate used.

Determination of enthalpies of protonation of benzenesulfonamide and CL 11.366

Hydrochloric acid solutions were reacted with solutions of sodium salts of benzenesulfonamide and CL 11.366. After applying the appropriate corrections to the calorimetrically measured values, the enthalpies of protonation (ΔH_s) were obtained.

Measurements on benzenesulfonamide were made with a LKB 8700 precision calorimeter. The calorimeter vessel was charged with 100 ml of 0.05 M benzenesulfon-

amide solution which had been partly neutralized with NaOH (pH = 10.70; pK_a = 10.1). The glass ampoule contained about 1 mmole of HCl at a concentration level of 1.6 M HCl. The pH was measured (± 0.01 pH unit) before and after the calorimetric reaction. In separate blank experiments, the enthalpies of dilution of the HCl samples were determined.

The enthalpy of protonation of the CL 11.366 salt was measured in the LKB batch microcalorimeter. In these experiments 4 ml of a 0.004 M sodium buffer of CL 11.366 (pH = 8.4; pK_a = 8.25) were reacted with 2 ml 0.002 M HCl.

Units of measurements. Results of the calorimetric measurements are expressed in terms of joules (J); 1 cal = 4.1840 J.

RESULTS

Human carbonic anhydrase C

There were several sources of error discovered during the work with the human carbonic anhydrase in the batch calorimeter. The enthalpies of dilution measurements of the enzyme resulted in substantial but scattered exothermic values. The effect was largely eliminated by treating all calorimeter solutions with a trace amount of the enzyme (to ensure equilibration of CO_2 with its hydrated form). With enzyme present in the buffer, the enthalpies of dilution were small and usually endothermic. However, even with this precaution results of the dilution measurements were more scattered than is usually encountered in this kind of calorimetric measurement. If the enzyme preparations had been stored for about two weeks, at 4 °C, significantly more exothermic ΔH values were found for the inhibition processes. For the measurements reported here the enzyme preparations were used within a week after they had been dialyzed.

Table I shows the results of measurements with benzene sulfonamide and the human C enzyme at 24.9 and 34.4 °C in 0.05 M HEPES buffer at pH 8.2.

TABLE I

ENTHALPIES OF BINDING (kJ·mole^{-1}) OF BENZENESULFONAMIDE TO HUMAN CARBONIC ANHYDRASE C IN 0.05 M HEPES BUFFER

24.9 °C, pH 8.20		34.4 °C, pH 8.08	
Reaction	Dilution	Reaction	Dilution
−44.94*	+0.38**	−43.64*	+1.42*
−44.77*	−0.32**	−43.18*	+0.88*
−47.53*		−43.72*	
−45.94**			
−45.79 ± 0.60*	+0.03 ± 0.30***	−43.51 ± 0.20***	+1.15 ± 0.30***
ΔH_{exp} = −45.8 ± 0.7		ΔH_{exp} = −44.7 ± 0.4	

* 0.147 mM human carbonic anhydrase C + 0.588 mM benzenesulfonamide + 1 μM human carbonic anhydrase C.

** 0.1506 mM human carbonic anhydrase C + 0.601 mM benzenesulfonamide + 1 μM human carbonic anhydrase C.

*** Uncertainties are estimates.

TABLE II

ENTHALPIES OF BINDING ($kJ \cdot mole^{-1}$) OF CL 11.366 TO HUMAN CARBONIC ANHYDRASE C IN 0.05 M HEPES BUFFER

25 °C, pH 8.20		35 °C, pH 8.10	
Reaction	Dilution	Reaction	Dilution
−62.26[a]	0.38[a]	−61.30[b]	2.76[b]
−63.01[a]	−0.32[a]	−63.18[b]	1.50[b]
−64.60[a]	0.17[b]	−61.09[b]	1.27[c]
−61.80[b]	1.05[b]	−64.35[b]	
−62.51[b]	1.27[c]	−61.46[b]	
−60.17[b]	0.36[c]	−62.22[c]	
		−63.89[c]	
−62.39 ± 0.60[d]	0.49 ± 0.20[d]	−62.50 ± 0.50[d]	1.84 ± 0.50[e]
$\Delta H_{exp} = -62.9 \pm 0.6$		$\Delta H_{exp} = -64.3 \pm 0.7$	

[a] 0.1506 mM human carbonic anhydrase C + 0.602 mM CL 11.366 + 1 μM human carbonic anhydrase C.

[b] 0.1473 mM human carbonic anhydrase C + 0.589 mM CL 11.366 + 1 μM human carbonic anhydrase C.

[c] 0.1478 mM human carbonic anhydrase C + 0.589 mM CL 11.366 + 1 μM human carbonic anhydrase C.

[d] Standard deviation of the mean.

[e] Uncertainty is an estimate.

TABLE III

RESULTS OF FLOW CALORIMETRIC EXPERIMENTS WITH BOVINE CARBONIC ANHYDRASE FORM B AT pH 8.2 AND 25 °C

	Inhibitor conc. (mM)	Buffer (0.05 M)	Bovine carbonic anhydrase B conc. (mM)	Molar ratio inhibitor/ enzyme	ΔH_{exp} ($kJ \cdot mole^{-1}$)
Benzene-sulfonamide	0.996	HEPES	0.249	1.99	−41.09
				3.00	−41.92
				4.00	−41.09
					−41.4 ± 0.3*
CL 11.366	0.996	HEPES	0.249	4.00	−60.3
Methazol-amide	0.996	HEPES	0.249	4.00	−60.7
Sulfanil-amide	0.62	Tris	0.1435	4.3	−45.56
					−44.60
					−45.1 ± 0.5*
p-Toluene-sulfonamide	0.615	Tris	0.1435	4.29	−45.2
Benzene-sulfonamide	0.574	Tris	0.1435	2.00	−40.92
					−41.34
				4.00	
					−41.1 ± 0.2*

* Uncertainties are estimates.

In Table II, results from corresponding measurements with CL 11.366 are summarized. Measurements were performed at 25 and 35 °C.

Bovine carbonic anhydrase B

In Tables III and IV the results of a number of determinations with the bovine enzyme in the flow calorimeter are shown. The enthalpies of dilution were measured and were used as corrections in the calculation of the enthalpy values shown in the tables.

The results from an inhibitor reaction which did not go to completion are shown in Table IV. KOCN was mixed in excess with bovine carbonic anhydrase in the

TABLE IV

RESULTS OF FLOW CALORIMETRIC BINDING EXPERIMENTS WITH BOVINE CARBONIC ANHYDRASE FORM B AND KOCN

Buffer: 0.05 M HEPES, pH 8.2; temperature: 25 °C; Q: the amount of heat evolved per mole of bovine carbonic anhydrase form B.

Bovine carbonic anhydrase B concn (mM)	Molar ratio KOCN/bovine carbonic anhydrase B	Q (kJ·mole^{-1})
0.249	1.992	17.41
0.249	4.00	21.30
0.249	19.92	30.71
0.249	40.0	31.42

flow calorimeter at increasing mole ratios. The apparent enthalpy values became increasingly exothermic with the mole ratio, but at a decreasing rate, changing very little between 20 and 40 mole KOCN/mole of enzyme.

Enthalpies of protonation of benzenesulfonamide and CL 11.366

In the experiments with benzenesulfonamide a significant quantity of the HCl was consumed by OH^-. A correction was applied using the results of the pH measurements and the heat of ionization value for water [8], 55.80 kJ·mole^{-1}. The results of the protonation experiments were further corrected for the enthalpy of dilution of the HCl. From the results of three experiments the enthalpy of protonation for benzenesulfonamide was derived to be $\Delta H_s = -34.81 \pm 0.04$ kJ·mole^{-1}.

In the experiments with CL 11.366 the HCl sample was quantitatively consumed by the sulfonamide salt. From four experiments the value $\Delta H_s = -26.5 \pm 0.3$ kJ·mole^{-1} was derived.

DISCUSSION

Binding of sulfonamides to carbonic anhydrase

The catalytic activity and the binding of anionic inhibitors as well as of sulfonamides depend on the ionization state of one group in the active site of carbonic anhydrase having a pK_a near 7 [1]. The chemical nature of this group is not known with certainty but all available data are in accordance with a model involving the

formation at high pH of a metal-coordinated OH^-. In terms of this Zn^{2+}–OH^- model the pH dependence of the binding of inhibitors can be described as a competition for a coordination site between OH^- and the anionic form of the inhibitor. In the case of inhibitors such as many sulfonamides and CN^- and others for which the acidic, unionized forms are predominant at neutral pH, it has been shown that the most important kinetic pathway for the formation of the complex involves a direct combination between the neutral form of the inhibitor and the basic form of the active site [10]. There is evidence, however, that in the complex, sulfonamides are bound in their ionized form [11].

In the following discussion we have treated the data in terms of the Zn^{2+}–OH^- model but the calculations are, of course, independent of any assumption about the chemical identity of the catalytic group. As a reference for the enzyme–sulfonamide binding reaction we may choose

$$(E\text{–}OH^- + NH_2SO_2R \rightleftharpoons E\text{–}\bar{N}HSO_2R + H_2O)_{aq} \tag{1}$$

for which the enthalpy change is ΔH°.

Under the conditions of the calorimetric measurements, however, it is the following process which is believed to take place:

$$(\beta E\text{–}OH_2 + (1-\beta)E\text{–}OH^- + \alpha\bar{N}HSO_2R + (1-\alpha)NH_2SO_2R + (\alpha-\beta)BH^+ \rightarrow E\text{–}\bar{N}HSO_2R + H_2O + (\alpha-\beta)B)_{aq} \tag{2}$$

where β is the degree of protonation of the enzyme and α is the degree of proton dissociation from the sulfonamide. B is the basic form of the buffer.

In order to obtain ΔH°, contributions from the following protonation reactions must be taken into account

$$(\alpha\bar{N}HSO_2R + \alpha H^+ \rightarrow \alpha NH_2SO_2R)_{aq} \tag{3}$$

$$(\beta E\text{–}OH^- + \beta H^+ \rightarrow \beta E\text{–}OH_2)_{aq} \tag{4}$$

and

$$(\alpha-\beta)B + (\alpha-\beta)H^+ \rightarrow (\alpha-\beta)BH^+ \tag{5}$$

for which the enthalpy changes are $\alpha\Delta H_S$, $\beta\Delta H_E$ and $(\alpha-\beta)\Delta H_B$, respectively.

From Eqns 1–5 an expression for ΔH° can be derived.

$$\Delta H^\circ = \Delta H_{exp} + \alpha(\Delta H_B - \Delta H_S) + \beta(\Delta H_E - \Delta H_B) \tag{6}$$

where ΔH_{exp} is the enthalpy change for Eqn 2.

In Tables V and VI the ΔH° values as well as some of the parameters used in the calculations are listed.

Values for ΔH_B were taken to be $-47.50\,kJ\cdot mole^{-1}$ and $-21.0\,kJ\cdot mole^{-1}$ for Tris [12] and HEPES [13], respectively.

TABLE V

CALCULATION OF ΔH° AND ΔC_p° FOR INHIBITOR COUPLING REACTIONS WITH HUMAN CARBONIC ANHYDRASE C

0.05 M HEPES buffer; $\beta = 0.06$.

Inhibitor	Temp. °C	pH	pK_a (sulfonamide)	α	ΔH° (kJ·mole^{-1})	ΔC_p (kJ·K^{-1}·mole^{-1})
Benzene-	24.90	8.20	10.1*	0.012	−45.7 ± 0.7	
sulfonamide	34.40	8.08	9.9	0.015	−44.5 ± 0.3	−0.13 ± 0.08
CL 11.366	25.00	8.20	8.25**	0.471	−60.3 ± 0.7	
	35.00	8.06	8.10	0.477	−61.7 ± 0.8	−0.14 ± 0.10

* Estimate based on a Hammet's $\sigma = 0$ and known pK_a values for sulfanilamide (pK_a = 10.7 [14]) and *p*-nitrobenzenesulfonamide (pK_a = 9.3 [9]).
** Ref. 15.

In Table VI the ΔC_p values for the inhibitor reactions with human C enzyme are also given,

$$\frac{\Delta(\Delta H)}{\Delta T} = \Delta C_p.$$

All pH measurements were performed at 24–25 °C and were used directly for the calculation of α at 25 °C. For the calorimetric experiments around 35 °C the pH values were estimated using the van 't Hoff equation. Literature values for the pK_a of the sulfonamides are all referred to 25 °C. For the calculation of α-values at 35 °C corrections were applied. At pH 8.2, α is very small for benzenesulfonamide and its simple derivatives (see Table VI) whereas it is large for methazolamide and Cl 11.366. ΔH_S values for benzenesulfonamide and CL 11.366 were determined in the present work. For the other sulfonamides (methazolamide, sulfanilamide and *p*-toluenesulfonamide) ΔH_S values were estimated (Table VI) using the experimentally determined ΔH_S values and assuming a linear relationship between pK_a and ΔH_S (cf. ref. 17). The

TABLE VI

CALCULATION OF ΔH° FOR INHIBITOR COUPLING REACTIONS WITH BOVINE CARBONIC ANHYDRASE FORM B

pH = 8.20; t = 25 °C; $\beta = 0.05$.

Inhibitor	Buffer	pK_a (sulfonamide)	α	ΔH_S (kJ·mole^{-1})	ΔH° (kJ·mole^{-1})
Benzenesulfonamide	HEPES	10.1	0.012	−34.81	−41.2 ± 0.3
	Tris	10.1	0.012		−41.4 ± 0.2
p-Toluenesulfonamide	Tris	10.25[a]	0.009	−35.6[d]	−45.2
Sulfanilamide	Tris	10.7[b]	0.003	−37.7[d]	−45.2 ± 0.5
Methazolamide	HEPES	7.4[c]	0.863	−22.6[d]	−59.0
CL 11.366	HEPES	8.25	0.471	−26.5	−57.7

[a] Estimate based on $\sigma = -0.17$ (cf. note * in Table V).
[b] Ref. 14.
[c] Ref. 16.
[d] Estimated, cf. text.

temperature variation for the ΔH_S values can be expected to be insignificant for the present calculations and was, therefore, not taken into account.

β-Values are small but are only known approximately since they were obtained from pH-rate profiles using *p*-nitrophenyl acetate as substrate. In the calculations the values 0.06 (pK = 6.9 [18])and 0.05 (pK = 7.0; Steiner, H., personal communication) were used for the bovine and for the human enzyme, respectively.

Pocker and Stone [19] have estimated the enthalpy value for the protonation of E–OH$^-$, ΔH_E, to be -29 kJ·mole^{-1}. This value, which was used in the present calculations was based on the temperature dependence of the esterase activity of the bovine enzyme. Both β and ΔH_E are judged to be rather uncertain, and it is clear that the derived values for the second correction term in Eqn 6 are approximate. However, this latter term appears to be very small, approx. -0.5 and 1.0 kJ·mole^{-1} for measurements in HEPES buffer and in Tris buffer, respectively.

Inspection of Table VI shows that the ΔH° values obtained for benzenesulfonamide with the bovine enzyme are identical in Tris and HEPES buffer. This supports the view that the buffer substances used do not bind to the active site or interfere in other ways with the protein–inhibitor binding process.

For benzenesulfonamide and CL 11.366 measurements were made with both the human C enzyme and the bovine enzyme. It is seen that for benzenesulfonamide ΔH° for the reaction with the human enzyme is about 4kJ·mole^{-1} more exothermic than with the bovine enzyme. For CL 11.366 the difference is in the same direction but slightly smaller.

For benzenesulfonamide and its simple derivatives equilibrium constants corresponding to Eqn 1 are accurately known and ΔG°, ΔH° and ΔS° values have been summarized for these reactions in Table VII. It is seen that they show the same pattern

TABLE VII

THERMODYNAMICS OF SOME CARBONIC ANHYDRASE INHIBITOR BINDING REACTIONS AT 25 °C, $(E\text{–}OH^- + NH_2SO_2R \rightleftharpoons E\text{–}\tilde{N}HSO_2R + H_2O)_{aq}$

Enzyme	Inhibitor	K	ΔG° (kJ·mole^{-1})	ΔH° (kJ·mole^{-1})	ΔS° (J·K^{-1}·mole^{-1})
Human C	Benzenesulfonamide	$2.2 \cdot 10^6$*	−36.1	−45.7	−32
Bovine form B	Benzenesulfonamide	$1.6 \cdot 10^6$**	−35.4	−41.3	−20
	Sulfanilamide	$3.4 \cdot 10^5$*	−31.6	−45.2	−46
	p-Toluensulfonamide	$3.3 \cdot 10^6$*	−37.2	−45.2	−26

* Lindskog, S., unpublished.
** Calculated from data in ref. 20.

of enthalpy driven reactions which are opposed by a small or moderate entropy change. A similar picture has emerged earlier from equilibrium measurements by Taylor et al. [9] on *p*-nitrobenzene sulfonamide, $\Delta G = -43$ kJ·mole^{-1}; $\Delta H = -40$ kJ·mole^{-1}; $\Delta S =$ 13 J·K^{-1} mole^{-1}. Their equilibrium measurements were made in phosphate buffer at pH 7.6 over the temperature range 15–40 °C.

The detailed structure of the particular sulfonamide–enzyme complexes studied here are not known. However, there is strong evidence [2, 11] that the $\tilde{N}HSO_2$-group

binds to the zinc ion in the active site cavity. It can further be assumed that hydrogen bonds are formed between the protein material and the inhibitor and that there are a great number of van der Waals interactions.

In discussions of the thermodynamics of biochemical binding processes, it is of vital importance to consider the role of the water. For instance, in the reaction of Eqn 1 the inhibitor is initially solvated by water thereby imposing certain structural changes on the bulk water. In the binding process the inhibitor is transferred into the deep cavity of the active site and the "structured water" originally surrounding the inhibitor will be transformed to the bulk water. From results of X-ray studies [2] it is further believed that about eight water molecules are displaced from the active site by the inhibitor. These water molecules will thus be transferred to the bulk water.

As yet it is not possible to make any detailed correlation between the thermodynamic quantities and the assumed structural features of the binding process. However, a few general conclusions can be drawn.

The ΔC_p° values derived for the binding of benzenesulfonamide and CL 11.366 are unusually small compared to most other ΔC_p values found for the binding of low molecular weight inhibitors to the active site of proteins, see e.g. ref. 21. Typical values for these processes are in the range of -1 to -2 kJ·K^{-1}·mole^{-1} for partially hydrophobic inhibitor molecules. On the other hand, the binding of hydrophilic saccharide inhibitors (GlcNAc, $(GlcNAc)_3$) to lysozyme has been shown to be accompanied by nearly zero ΔC_p values [22]. The large negative ΔC_p values usually found have been interpreted as due to a decrease in the exposure to water of non-polar moieties of the protein and/or of the inhibitor or resulting from a stiffening of the protein structure.

From the results of recent calorimetric measurements [23] ΔC_p for the transfer of a phenyl group from water to a nonaqueous phase is estimated to be about -0.2 kJ·K^{-1}·mole^{-1}. One may, therefore, expect a ΔC_p contribution of this order for the transfer of the simple aromatic sulfonamides from bulk water to the binding site of the enzyme.

It is difficult to estimate the ΔC_p contribution for the displacement of eight water molecules from the active site. The bound water may be thought of as having a low C_p value similar to that of ice or that found for many hydrates [24] in which case one would expect a ΔC_p contribution of about $+0.3$ kJ·K^{-1}·mole^{-1}. On the other hand, results from recent C_p determinations of water absorbed to solid proteins [25] suggest bound water to have an apparent heat capacity which is about 30% higher than that of bulk water.

Results from X-ray studies [2] as well as other data [11] indicate that the sulfonamide–enzyme binding process does not involve a large change in conformation of the protein molecule, which observations are in general agreement with the low values found for ΔC_p, as well as ΔS, of the binding process.

We may formally consider the reference reaction 1 as an overall process composed of the ionization of the sulfonamide and a simple displacement reaction

$$(\text{E–OH}^- + \bar{\text{N}}\text{HSO}_2\text{R} \rightarrow \text{E–}\bar{\text{N}}\text{HSO}_2\text{R} + \text{OH}^-)_{aq} \qquad (7)$$

for which the enthalpy change, ΔH°, equals

$$\Delta H_7^\circ = \Delta H^\circ + \Delta H_S + \Delta H_I \qquad (8)$$

where ΔH_I is the enthalpy of ionization of water [8], 55.80 kJ·mole^{-1}.

ΔH_7° (25 °C) for the human C enzyme with benzenesulfonamide and CL 11.366 in HEPES are thus derived to be -2.47 and -31.0 kJ·mole^{-1}, respectively. For the other compounds, ΔH_7° values are of the same magnitude although calculations in these cases are less accurate because the ΔH_S values available are only estimates. The fact that ΔH_7° values are similar for the different sulfonamides investigated suggests that the binding of the $\bar{N}HSO_2$-group provides the dominant contribution to the enthalpy change.

The standard free energy change for reaction 7, ΔG_7°, is given by

$$\Delta G_7^\circ = \Delta G^\circ - RT \ln \frac{K_w}{K_a} \tag{9}$$

where K_w is the ion product for water and K_a is the ionization constant for the sulfonamide. For benzenesulfonamide and the human C enzyme $\Delta G_7^\circ = -13.8$ kJ·mole^{-1}. The corresponding entropy change is $\Delta S_7^\circ = -36$ J·mole^{-1}·K^{-1}.

Binding of cyanate to the bovine enzyme

The results in Table IV refer to the reaction

$$(E_t + OCN^- \rightleftharpoons E\text{–}OCN^-)_{aq} \tag{10}$$

where E_t represents the total bovine enzyme irrespective of the ionization state of the catalytic group. A quadratic equation in terms of equilibrium constant and enthalpy change was written for every set of concentration and heat data. By means of a computer program all equations were plotted on the same graph and the best intersection of all curves was chosen as the simultaneous solution. The results obtained for the equilibrium constant and enthalpy change were as follows: $K_{exp} = 4.5 \cdot 10^3$ M^{-1} and $\Delta H_{exp} = -32.8$ kJ·mole^{-1}. The K-value compares favorably with those derived from studies of the inhibition of the esterase activity of the enzyme. Interpolation of the values obtained by Thorslund and Lindskog [18] gives $K_{exp} = 7 \cdot 10^3$ M^{-1} at pH 8.2.

Under the conditions of the calorimetric experiments, and using the Zn^{2+}–OH^- model, the stoichiometry of the reaction with OCN^- can be assumed to be as follows:

$$\beta E\text{–}OH_2 + (1-\beta)E\text{–}OH^- + OCN^- + (1-\beta)BH^+ \rightleftharpoons E\text{–}OCN^- + (1-\beta)B + H_2O \tag{11}$$

By applying corrections similar to those used for the sulfonamide a value of $\Delta H_{12}^\circ = +1.6$ kJ·mole^{-1} is obtained for the reaction

$$E\text{–}OH^- + OCN^- \rightleftharpoons E\text{–}OCN^- + OH^- \tag{12}$$

which corresponds to Eqn 7. Similarly, the value of ΔG° (kJ·mole^{-1}) for Eqn 12 is given by the following:

$$\Delta G_{12}^\circ = \Delta G_{exp}^\circ - 5.711\,(pH - pK_w - \log(1-\beta)) \tag{13}$$

Using ΔG_{exp}° calculated from K_{exp} a value of $\Delta G_{exp}^\circ = +12.2$ kJ·mole^{-1} is obtained. The value of ΔS_{12}° would thus be -36 J·K^{-1}·mole^{-1}, which is identical to the value of ΔS_7° obtained for benzenesulfonamide and the human C enzyme. We

thus conclude that the stronger binding of sulfonamides compared with OCN^- reflects a difference in the enthalpy of binding. It remains to be seen whether this principle applies also to other anionic inhibitors of carbonic anhydrase.

ACKNOWLEDGEMENTS

This work has been supported by grants from the Swedish Natural Science Research Council (2131, 2296-017) and from the Swedish Medical Research Council (B73-13x-2722-05B). The computer program was written by J. C. Foster. The technical assistance by Mrs E. Qvarnström, Miss A.-C. Carlsson and Miss E.-K. Gandvik is gratefully acknowledged.

REFERENCES

1 Lindskog, S., Henderson, L. E., Kannan, K. K., Liljas, A., Nyman, P. O. and Strandberg, B. (1971) in The Enzymes, 3rd edn (Boyer, P. D., ed.), Vol. 5, pp. 587–665, Academic Press, New York and London
2 Bergstén, P.-C., Waara, I., Lövgren, S., Liljas, A., Kannan, K. K. and Bengtsson, U. (1972) in Oxygen Affinity of Hemoglobin and Red Cell Acid Base Status (Rørth, M. and Astrup, P., eds), pp. 363–383, Munksgaard, Copenhagen and Academic Press, New York
3 Lindskog, S. (1960) Biochim. Biophys. Acta 39, 218–226
4 Henderson, L. E. and Henriksson, D. (1973) Anal. Biochem. 51, 288–296
5 Nyman, P. O. and Lindskog, S. (1964) Biochim. Biophys. Acta 85, 141–151
6 Wadsö, I. (1968) Acta Chem. Scand. 22, 927–937
7 Monk, P. and Wadsö, I. (1968) Acta Chem. Scand. 22, 1842–1852
8 Sober, H. A. (1968) Handbook of Biochemistry, p. J-135, The Chemical Rubber Co., Cleveland
9 Taylor, P. W., King, R. W. and Burgen, A. S. V. (1970) Biochemistry 9, 2638–2645
10 Taylor, P. W. and Burgen, A. S. V. (1971) Biochemistry 10, 3859–3866
11 King, R. W. and Burgen, A. S. V. (1970) Biochim. Biophys. Acta 207, 278–285
12 Hill, J. O., Öjelund, G. and Wadsö, I. (1969) J. Chem. Thermodyn. 1, 111–116
13 Beres, L. and Sturtevant, J. M. (1971) Biochemistry 10, 2120–2126
14 Lindskog, S. (1969) in CO_2: Chemical, Biochemical and Physiological Aspects (Forster, R. E., Edsall, J. T., Otis, A. B. and Roughton, F. J. W., eds), NASA SP-188, pp. 157–165, Washington, D.C.
15 Lindskog, S. and Thorslund, A. (1968) Eur. J. Biochem. 3, 453–460
16 Lindskog, S. (1963) J. Biol. Chem. 238, 945–951
17 Eberson, L. and Wadsö, I. (1963) Acta Chem. Scand. 17, 1552–1562
18 Thorslund, A. and Lindskog, S. (1967) Eur. J. Biochem. 3, 117–123
19 Pocker, Y. and Stone, J. T. (1968) Biochemistry 7, 4139–4145
20 Kernohan, J. C. (1966) Biochim. Biophys. Acta 118, 405–412
21 Wadsö, I. (1972) in MTP International Review of Science, Phys. Chem., Ser. One (Skinner, H. A., ed.), Vol. 10, pp. 1–43, Butterworths, London and University Park Press, Baltimore
22 Bjurulf, C. and Wadsö, I. (1972) Eur. J. Biochem. 31, 95–102
23 Nichols, N. and Wadsö, I., to be published
24 Rossini, F. D., Wagman, D. D., Evans, W. H., Levine, S. and Jaffe, J. (1952) Selected Values of Chemical Thermodynamic Properties, Circular of the National Bureau of Standards 500, U.S. Government Printing Office, Washington, D.C.
25 Suurkuusk, J. (1974) Acta Chem. Scand., in the press