Thermodynamics of hydrogen cyanide and hydrogen fluoride binding to cytochrome *c* peroxidase and its Asn-82→Asp mutant

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The thermodynamics of binding of fluoride and cyanide to cytochrome *c* peroxidase (CCP) and its Asn-82→Asp mutant (D82CCP) in phosphate and acetate buffer at an ionic strength of 0.15 mol·kg⁻¹ from pH 5.0 to 7.1 were investigated by titration calorimetry at 289 and 297 K. The binding reactions are enthalpically driven. The fluoride-binding constants determined from the titration calorimetry results were in agreement with those determined from difference-spectroscopy measurements. For cyanide binding to CCP at 297.9 K, the binding constant decreased from $8.95(\pm 0.83) \times 10^5 \text{ M}^{-1}$ at pH 7.0 to $4.04(\pm 0.23) \times 10^5 \text{ M}^{-1}$ at pH 5.0, and the binding enthalpy increased from $-57.2 \pm 1.4 \text{ kJ} \cdot \text{mol}^{-1}$ at pH 7.0 to

$-48.6 \pm 1.8 \text{ kJ} \cdot \text{mol}^{-1}$ at pH 5.0. For fluoride binding to CCP, the binding constant increased from $8.41(\pm 0.54) \times 10^3 \text{ M}^{-1}$ at pH 7.0 to $3.11(\pm 0.09) \times 10^5 \text{ M}^{-1}$ at pH 5.0 and the binding enthalpy increased from $-71.9 \pm 1.1 \text{ kJ} \cdot \text{mol}^{-1}$ at pH 7.0 to $-67.0 \pm 1.9 \text{ kJ} \cdot \text{mol}^{-1}$ at pH 5.0. The binding enthalpies for D82CCP were about the same as those for CCP. However, the binding constants for cyanide and fluoride to D82CCP were respectively a factor of two less and at least an order of magnitude less than the corresponding binding constants of CCP. Decreased ligand-binding strength in the D82CCP mutant is thus entirely due to entropic effects.

INTRODUCTION

The ferric haem of yeast cytochrome *c* peroxidase (CCP) (EC 1.11.1.5) reacts rapidly (rate constant $\geq 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$) and efficiently with H₂O₂ to yield a two-electron oxidized enzyme intermediate commonly termed Compound I:

The reaction cycle is completed via reduction of Compound I by reducing equivalents supplied by ferrous cytochrome *c*. The geometry of the ferric-ion-binding site on CCP is clearly optimized for peroxide binding followed by essentially irreversible heterolytic O–O cleavage. In common with other ferric haem proteins, a number of other small ligands such as HF and HCN also bind, but reversibly, to the ferric iron. As the structural and thermodynamic factors governing peroxide binding to CCP cannot be elucidated, research on the binding mechanism has focused on the binding of HF, HCN and other small ligands to CCP.

The structures of the enzyme complexed with fluoride and cyanide have been determined by X-ray crystallography to a resolution of 1.85 Å (0.185 nm) [1]. Differences between the complexed and uncomplexed structures show that ligand binding involves significant movement of Arg-48 and His-52 as well as rearrangement of water molecules in the distal haem pocket where the binding occurs. Hydrogen bonds are formed between the ligand and distal residues Trp-51 and His-52, which is also hydrogen-bonded to Asn-82. The His-52–Asn-82 interaction is evident in the lignin peroxidase structure [2], and is predicted to be conserved in other peroxidases as well [3]. Edwards and Poulos [1] proposed that this interaction controls the proton-accepting and -donating properties of His-52. Thus determining the effect of changing Asn-82 to Asp (D82CCP) on the thermo-

dynamics of ligand binding is important. In addition, Arg-48 moves 0.3 Å away from the bound cyanide ligand but 2.0 Å towards the bound fluoride [1]. Movements of Arg-48 and His-52 on ligand binding are predictable from the CCP catalysis mechanism proposed by Poulos and co-workers [4–6] which describes His-52 and Arg-48 as working in concert to stabilize the charge separation required in the heterolytic fission of the peroxide O–O bond. In the present investigation, the thermodynamics of binding HCN and HF to CCP and its D82CCP mutant are determined in terms of not only the association constants which have been determined for CCP but also the binding enthalpy and entropy differences, ΔH_b and ΔS_b respectively.

The thermodynamics of binding of cyanide and fluoride to CCP have been determined in terms of the binding constant, K_b , for the binding reaction,

$$K_b = [\text{CCP-HX}]/[\text{CCP}][\text{HX}] \quad (2b)$$

where [CCP] and [HX] are the free enzyme and free ligand concentrations respectively. Utilizing stopped-flow spectroscopy to investigate the binding of HCN and HF to CCP, Erman [7,8] showed that the dependence of the association rate constants on pH involves ionization of the ligand and a group on the enzyme with a pK_a near 5.5, whereas the pH dependence of the dissociation constant involves ionization of two ionizable groups in the complex with pK_a values in the 6.0–6.5 and 7–8 ranges. K_b yields the Gibbs energy change (ΔG_b^0) as $\Delta G_b^0 = -RT \ln(K_b)$ where $R = 8.31451 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ and T is the temperature in K. In this investigation, the thermodynamic parameters of the binding of HCN and HF to the CCP haem iron as a function of pH and temperature in buffer at an ionic strength of

Abbreviation used: CCP, cytochrome *c* peroxidase.

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$0.15 \text{ mol} \cdot \text{kg}^{-1}$ are determined from titration calorimetry and u.v. spectroscopy measurements.

EXPERIMENTAL

Materials

The cloning and expression of CCP and D82CCP have been described previously [9]. The purity index of CCP and mutant, which is the ratio A_{408}/A_{280} , ranged from 1.25 to 1.31 in 0.1 M potassium phosphate buffer at pH 6.0 and 298 K, in agreement with results previously reported by Yonetani and co-workers [10,11]. Absorbance was determined using a Perkin-Elmer 4B u.v./visible spectrophotometer. Potassium phosphate, phosphoric acid, sodium phosphate, sodium monophosphate, KF, acetic acid, potassium acetate and KCN were reagent grade. Deionized water was used in all experiments.

Preparation of solutions

Solutions of CCP were dialysed overnight at 278 K in either phosphate or acetate buffer at the appropriate pH adjusted to $0.15 \text{ mol} \cdot \text{kg}^{-1}$. pH was measured with an Orion 811 pH-meter equipped with a Corning EX-L glass electrode. Concentrations of CCP solutions were determined spectrophotometrically using an absorption coefficient of 99 mM^{-1} at 408 nm and a molecular mass of 34168 daltons [12,13]. KF and KCN ligand solutions at the millimolar level were prepared by weight in the dialysate in order to match the solvent of the protein and ligand solutions as closely as possible. The pH of the ligand solution was measured and adjusted to the pH of the CCP solutions.

Titration-calorimetry measurements

Titration-calorimetry measurements were performed using a Microcal Omega titration calorimeter as described previously [14–17]. Briefly, the titration calorimeter consists of a 1.374 ml cell containing the protein solution and a matched reference cell containing the dialysate in an adiabatic enclosure. Samples of the ligand solution were added via a rotating stirrer-syringe operated with a plunger driven by a stepping motor. Heat absorbed or released on addition of ligand solution to protein solution was measured by a thermoelectric sensor between the 1.374 ml cell and the reference cell. Ligand concentrations were about $20 \times [\text{CCP}]$ or $[\text{D82CCP}]$ and the enzyme solutions were 0.06–0.38 mM in order to keep the product K_b (CCP or D82CCP) below 1000 to minimize error in K_b determinations [14]. Total heat, Q_t , was then fitted via a non-linear least-squares minimization method [16] to the total ligand concentration, $[\text{HX}]_t$, using the following equation [14]:

$$Q_t = n[\text{CCP}]_t \Delta H_b V (1 + [\text{HX}]_t / n[\text{CCP}]_t + 1/nK[\text{CCP}]_t) - \{(1 + [\text{HX}]_t / n[\text{CCP}]_t + 1/nK[\text{CCP}]_t)^2 - 4[\text{HX}]_t / n[\text{CCP}]_t\}^{1/2} \quad (3)$$

where n is the number of sites, i.e. the stoichiometry, $[\text{CCP}]_t$ is the total protein concentration and V is the cell volume. Each titration-calorimetry scan yields n , ΔH_b and K_b and values for ΔS_b were obtained from the following equation:

$$-nRT \ln(K_b) = \Delta G_b^0 = \Delta H_b - T\Delta S_b \quad (4)$$

Spectrophotometric-difference measurements

As fluoride binding to D82CCP was observed to be weak at pH 6.0 and above and the attainable concentration of mutant was too low ($< 0.10 \text{ mM}$) for accurate analysis by titration

calorimetry, the binding constant of fluoride was determined from spectrophotometric-difference measurements. The measurements were performed using a Shimadzu UV-265 spectrophotometer thermostatically controlled at $298.2 \pm 0.2 \text{ K}$ or a Cary 4 spectrophotometer thermostatically controlled at 287.0, 293.0 and 298.0 K. Buffer was added to both sample and reference cells and an additional 25–40 μl of CCP or D82CCP solution was added to the sample cell to yield final protein concentrations 5–10 μM . A_{407} was recorded and then 5–20 μl portions of the fluoride solution were added to both the sample and reference cells. On addition of fluoride to the protein solution, the Soret absorbance increased and the Soret maximum (407 nm) shifted to a slightly shorter wavelength (404 nm). The total change in absorbance at saturation, ΔA_s , the change in absorbance below saturation, ΔA , $[\text{CCP}]_t$ and $[\text{HX}]_t$ yield the equilibrium concentrations of the complex, ligand, and CCP [2]

$$[\text{CCP-HX}] = \Delta A / \Delta A_s [\text{CCP}]_t \quad (5a)$$

and as

$$K_b = [\text{CCP-HX}] / ([\text{CCP}]_t - [\text{CCP-HX}]) ([\text{HX}]_t - [\text{CCP-HX}]) \quad (5b)$$

K_b can then be determined from the slope of a Scatchard plot of $[\text{CCP-HX}] / ([\text{HX}]_t - [\text{CCP-HX}])$ versus $[\text{CCP-HX}]$.

RESULTS

Titration-calorimetry measurements

Typical titration-calorimetry measurements made by adding 5 μl of ligand solution in phosphate buffer to CCP in phosphate buffer are shown in Figures 1 and 2. In Figure 1(a), 5 μl portions of 2.3 mM KCN (pH 5.0) were added to 0.08 mM CCP at 298.0 K and the area of the titration peaks decreased from 0.582 to 0.017 mJ. In Figure 2(a), 5 μl portions of 7.5 mM KF (pH 7.0) were added to 0.17 mM CCP at 298.0 K and the area of the

Figure 1 (a) Calorimetric titration of 5 μl portions of 2.3 mM KCN in phosphate buffer added to 0.08 mM CCP solution at pH 5.0 and 298.0 K and (b) binding isotherm of the titration shown in (a)

Figure 2 (a) Calorimetric titration of 5 μl portions of 7.5 mM KF in phosphate buffer added to 0.17 mM CCP solution at pH 7.0 and 298.0 K and (b) binding isotherm of the titration shown in (a)

titration peaks decreased from 1.35 mJ to 0.10 mJ. The titrations were continued until the areas were less than 10% of the initial peak area. Heat was not observed on titrating the ligand solution into the buffer solution, thus indicating that there is no contribution from the heat of dilution of the ligand solution. Fits of the total heat as a function of total ligand concentration to eqn. (3) are shown in Figures 1(b) and 2(b), and the results are presented in Tables 1 and 2 along with the results of measurements obtained with D82CCP. The stoichiometries of the binding reactions for CCP and D82CCP average 0.8 ± 0.1 for fluoride binding and 0.9 ± 0.1 for cyanide binding from fits of the data to eqn. (3). The stoichiometries were based on the assumption that the total enzyme concentration determined from spectroscopic measurements at 408 nm is free to bind with the ligand and that the ligand solutions prepared by weight were correct. As the heats of binding at 288 and 298 K are within experimental error, the heat-capacity change for the binding reactions, ΔC_p , is negligible.

In the titration-calorimetry measurements, heats of binding at high binding affinities and high ligand and protein concentrations (i.e. $K_b \geq 0.5 \times 10^6 \text{ M}^{-1}$ at $\geq 0.08 \text{ mM}$ CCP and millimolar ligand concentration) can be determined with more than 96% accuracy from the ratio of the first few titration peak areas to the amount of ligand added for each titration to the protein solution [17]. This method of analysis is applicable to HF binding at pH 5.0 and to HCN binding at pH 5.0–7.0. The heats of binding determined by this method, which does not depend on accurate values of the CCP concentration, are in good agreement with values of ΔH_b determined from fits to eqn. (3), although the stoichiometry of the binding reactions is less than one.

The determinations of K_b for fluoride binding are in agreement with the determinations of K_b obtained from difference spectroscopy at pH 5 for D82CCP and at pH 6 and 7 for CCP in Table 2. The difference-spectroscopy analysis is based on a Scatchard plot of the increase in the Soret band of CCP at 407 nm as a function of added ligand concentration. Difference spectroscopy measurements on CCP and D82CCP at pH 7.1 were also performed at 287.0, 293.0, and 298.0 K for determination of the binding enthalpy and entropy and are presented in Table 3. Van't Hoff plots of $\ln(K_b)$ versus $1/T$ of the binding constants determined at pH 7.1 for CCP and D82CCP are shown in Fig. 3. The enthalpy for HF binding to CCP is $-56.5 \pm 0.8 \text{ kJ} \cdot \text{mol}^{-1}$ in Table 3, which is higher than the HF-binding enthalpies determined from titration calorimetry in Table 2. The HF-binding enthalpy of $-69.9 \pm 1.7 \text{ kJ} \cdot \text{mol}^{-1}$ for the mutant at pH 7.1 (Table 3) is close to the value of $-71.9 \pm 1.1 \text{ kJ} \cdot \text{mol}^{-1}$ for CCP from titration-calorimetry measurements at pH 7.0.

A comparison of the binding constants with values from the literature is presented in Table 4. The literature values were determined by the ratio of the association to dissociation rate constants [7,8] and, also, by the ratio of the equilibrium concentrations of complex, CCP and ligand for cyanide binding [8]. For cyanide binding, the literature values [8] are less than a factor of 2 within the titration-calorimetry values, whereas the literature values for fluoride binding [7] are about a factor of 5 less than the titration-calorimetry values above pH 5.0. Determination of the rate constants involves defining some kinetic scheme for the association and dissociation processes, and they are thus more susceptible to error. In fact, the literature values for the cyanide-binding constants exhibit a wide variation between the kinetically derived constants and the equilibrium-determined constants.

At 298 K, the cyanide-binding constant increases from $4.04(\pm 0.23) \times 10^5 \text{ M}^{-1}$ at pH 5.0 to $8.95(\pm 0.83) \times 10^5 \text{ M}^{-1}$ at pH 7.0, whereas the binding enthalpy decreases from $-48.6 \pm 1.8 \text{ kJ} \cdot \text{mol}^{-1}$ at pH 5.0 to $-57.2 \pm 1.4 \text{ kJ} \cdot \text{mol}^{-1}$ at pH 7.0. In contrast, as shown in Figure 4, the fluoride-binding

Table 1 Thermodynamic parameters of HCN binding to CCP and D82CCP mutant in phosphate buffer (I 0.15 $\text{mol} \cdot \text{kg}^{-1}$)

pH	Temperature (K)	K_b (M^{-1})	$-\Delta G_b^0$ ($\text{kJ} \cdot \text{mol}^{-1}$)	$-\Delta H_b$ ($\text{kJ} \cdot \text{mol}^{-1}$)	$-\Delta S_b$ ($\text{J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$)	No. of determinations
5.0	287.9 ± 0.1	$1.01(\pm 0.03) \times 10^6$	33.1 ± 0.1	47.4 ± 3.1	50 ± 3	4
5.0	297.9 ± 0.2	$4.04(\pm 0.23) \times 10^5$	32.0 ± 0.1	48.6 ± 1.8	56 ± 2	4
6.0	287.8 ± 0.2	$1.54(\pm 0.09) \times 10^6$	34.1 ± 0.1	63.3 ± 2.2	101 ± 2	4
6.0	297.8 ± 0.1	$5.80(\pm 0.52) \times 10^5$	32.9 ± 0.2	56.3 ± 1.1	79 ± 1	6
*6.0	298.6 ± 0.1	$2.83(\pm 0.03) \times 10^5$	31.1 ± 0.1	56.1 ± 0.5	84 ± 1	2
7.0	288.8 ± 0.9	$2.10(\pm 0.05) \times 10^6$	34.9 ± 0.1	61.7 ± 2.8	93 ± 3	4
7.0	297.8 ± 0.1	$8.95(\pm 0.83) \times 10^5$	34.0 ± 0.2	57.2 ± 1.4	78 ± 1	7

* Results of measurements performed on the D82CCP mutant.

Table 2 Thermodynamic parameters of HF binding to CCP and D82CCP mutant in phosphate buffer (I 0.15 mol $\cdot$ kg $^{-1}$)

pH	Temperature (K)	K_b (M $^{-1}$)	$-\Delta G_b^0$ (kJ $\cdot$ mol $^{-1}$)	$-\Delta H_b$ (kJ $\cdot$ mol $^{-1}$)	$-\Delta S_b$ (J $\cdot$ K $^{-1}$ $\cdot$ mol $^{-1}$)	No. of determinations
*4.5	298.2 $\pm$ 0.1	1.26 ($\pm$ 0.13) $\times 10^5$	34.8 $\pm$ 0.3			
*†4.5	298.2 $\pm$ 0.1	2.22 ($\pm$ 0.22) $\times 10^4$	24.8 $\pm$ 0.3			
5.0	288.1 $\pm$ 0.7	5.99 ($\pm$ 0.28) $\times 10^5$	31.9 $\pm$ 0.1	63.4 $\pm$ 3.8	109 $\pm$ 4	5
5.0	298.0 $\pm$ 0.1	3.11 ($\pm$ 0.09) $\times 10^5$	31.3 $\pm$ 0.1	67.0 $\pm$ 1.9	120 $\pm$ 2	4
†5.0	297.8 $\pm$ 0.1	1.12 ($\pm$ 0.03) $\times 10^4$	23.0 $\pm$ 0.2	77.7 $\pm$ 0.2	184 $\pm$ 1	2
*†5.0	298.0 $\pm$ 0.1	1.2 ($\pm$ 0.1) $\times 10^4$	23.3 $\pm$ 0.2			
*5.5	298.2 $\pm$ 0.1	2.7 ($\pm$ 0.3) $\times 10^5$	31.0 $\pm$ 0.3			
*†5.5	298.2 $\pm$ 0.1	6.5 ($\pm$ 0.7) $\times 10^3$	21.8 $\pm$ 0.3			
6.0	288.1 $\pm$ 0.2	1.25 ($\pm$ 0.01) $\times 10^5$	28.1 $\pm$ 0.1	77.0 $\pm$ 3.8	170 $\pm$ 4	4
6.0	297.9 $\pm$ 0.1	6.10 ($\pm$ 0.22) $\times 10^4$	27.3 $\pm$ 0.1	83.8 $\pm$ 3.6	190 $\pm$ 4	6
†6.0	298.0 $\pm$ 0.1	3.7 ($\pm$ 0.4) $\times 10^2$	14.6 $\pm$ 0.2			
*6.2	298.2 $\pm$ 0.2	5.21 ($\pm$ 0.5) $\times 10^4$	26.9 $\pm$ 0.3			
*†6.2	298.2 $\pm$ 0.2	5.10 ($\pm$ 0.51) $\times 10^2$	15.5 $\pm$ 0.3			
*6.6	298.2 $\pm$ 0.2	1.53 ($\pm$ 0.15) $\times 10^4$	23.9 $\pm$ 0.3			
*†6.6	298.2 $\pm$ 0.2	7.94 ($\pm$ 0.79) $\times 10^1$	10.8 $\pm$ 0.3			
7.0	287.9 $\pm$ 0.2	1.72 ($\pm$ 0.02) $\times 10^4$	23.4 $\pm$ 0.1	71.1 $\pm$ 3.3	166 $\pm$ 3	4
7.0	297.9 $\pm$ 0.4	8.41 ($\pm$ 0.54) $\times 10^3$	22.4 $\pm$ 0.2	71.9 $\pm$ 1.1	166 $\pm$ 1	7
*†7.0	298.0 $\pm$ 0.1	3.36 ($\pm$ 0.50) $\times 10^1$	8.2 $\pm$ 0.4			

* Results of difference spectroscopy measurements so that only the binding constant is reported.

† Results of measurements performed on the D82CCP mutant.

Table 3 Thermodynamic parameters of HF binding to CCP and D82CCP mutant in phosphate buffer (I 0.15 mol $\cdot$ kg $^{-1}$) at pH 7.1 from difference-spectroscopy measurements

	Temperature (K)	K_b (M $^{-1}$)	$-\Delta G_b^0$ (kJ $\cdot$ mol $^{-1}$)	$-\Delta H_b^*$ (kJ $\cdot$ mol $^{-1}$)	$-\Delta S_b^*$ (J $\cdot$ K $^{-1}$ $\cdot$ mol $^{-1}$)
CCP	287.0	1.73 ($\pm$ 0.10) $\times 10^4$	23.3 $\pm$ 0.1	56.5 $\pm$ 0.8	115 $\pm$ 16
CCP	293.0	1.09 ($\pm$ 0.06) $\times 10^4$	22.6 $\pm$ 0.1	56.5 $\pm$ 0.8	116 $\pm$ 16
CCP	298.0	7.19 ($\pm$ 0.43) $\times 10^3$	22.0 $\pm$ 0.1	56.5 $\pm$ 0.8	115 $\pm$ 16
D82CCP	287.0	66 $\pm$ 4	10.0 $\pm$ 0.1	69.9 $\pm$ 1.7	209 $\pm$ 5
D82CCP	293.0	35 $\pm$ 2	8.7 $\pm$ 0.1	69.9 $\pm$ 1.7	209 $\pm$ 5
D82CCP	298.0	21 $\pm$ 1	7.5 $\pm$ 0.1	69.9 $\pm$ 1.7	209 $\pm$ 5

* The binding enthalpy was determined from a van't Hoff plot of $\ln(K_b)$ versus $1/T$.

Figure 3 Plots of $\ln[K_b$ (M $^{-1}$)] versus $1/T$ for fluoride binding to CCP (●) and D82CCP (■) at pH 7.1

The K_b values were determined from difference-spectroscopy measurements.

constant decreases from $3.11(\pm 0.09) \times 10^5$ M $^{-1}$ at pH 5.0 to $8.41(\pm 0.54) \times 10^3$ M $^{-1}$ at pH 7.0, whereas the binding enthalpy decreases from -65.2 ± 1.8 kJ $\cdot$ mol $^{-1}$ to -71.5 ± 0.4 kJ $\cdot$ mol $^{-1}$.

The D82 mutant exhibits lower affinity for HF binding. For example, HF binding to D82CCP at pH 5.0 yields $K_b = 1.2(\pm 0.1) \times 10^4$ M $^{-1}$ ($\Delta G_b^0 = -23.0 \pm 0.2$ kJ $\cdot$ mol $^{-1}$) whereas binding to CCP yields $K_b = 3.11(\pm 0.09) \times 10^5$ M $^{-1}$ ($\Delta G_b^0 = -31.3 \pm 0.1$ kJ $\cdot$ mol $^{-1}$). For HCN binding at pH 6.0, the mutant binding constant is $2.83(\pm 0.03) \times 10^5$ M $^{-1}$, about half the CCP binding constant of $5.83(\pm 0.52) \times 10^5$ M $^{-1}$, whereas the binding enthalpy is the same [ΔH_b (D82CCP) = -56.1 ± 1.1 kJ $\cdot$ mol $^{-1}$ and ΔH_b (CCP) = -56.1 ± 0.5 kJ $\cdot$ mol $^{-1}$].

DISCUSSION

It has been shown that the association and dissociation rates of ligand binding to CCP are pH-dependent and result from protonation and deprotonation of amino acid residues in CCP [7,8]. This results in the observed pH-dependence of the binding constant shown in Tables 1 and 2 and, more specifically, in Figure 4. The lack of any dependence of the association rate on ionic strength over this ionic-strength and pH range [18] and the hydrogen-bonding pattern around the ligand from X-ray-crystallography studies of the CCP complexes indicate that the protonated form of the ligand binds to CCP [19]. Accordingly,

Table 4 Comparison of equilibrium constants with literature values for binding of fluoride and cyanide to CCP at / 0.15

Ligand	pH	Experimental K_b from titration calorimetry (M^{-1})	Temperature (K)	Literature values at 298.15 K* (M^{-1})
HCN	5.0	$4.04(\pm 0.23) \times 10^5$	297.9 ± 0.2	$1.3(\pm 0.4) \times 10^5$ – $3.3(\pm 3.3) \times 10^5$
HCN	6.0	$5.80(\pm 0.52) \times 10^5$	297.8 ± 0.1	$1.7(\pm 0.3) \times 10^5$ – $2.8(\pm 0.5) \times 10^5$
HCN	7.0	$8.95(\pm 0.83) \times 10^5$	297.8 ± 0.1	$1.3(\pm 0.1) \times 10^5$ – $5.0(\pm 1.5) \times 10^5$
HF	5.0	$3.11(\pm 0.09) \times 10^5$	298.0 ± 0.1	$7.1(\pm 1.1) \times 10^4$
HF	6.0	$6.10(\pm 0.22) \times 10^4$	297.9 ± 0.1	$1.7(\pm 1.1) \times 10^4$
HF	7.0	$8.41(\pm 0.54) \times 10^3$	297.9 ± 0.4	$1.7(\pm 0.6) \times 10^3$

* Literature values of cyanide binding are from ref. [8] and those for fluoride binding are from ref. [7].

† These values were determined from equilibrium concentrations of the complex, CCP and the ligand. All the other literature values were determined from the ratio of the association rate constant to the dissociation rate constant.

Figure 4 Plot of $\ln[K_b (M^{-1})]$ versus pH for fluoride binding to CCP (●) and D82CCP (■)

The ⊗ points are the titration-calorimetry results.

Figure 5 Schematic of the interactions at the binding site where $X = F^-$ or CN^- from ref. [1]

only the protonated form of the ligand will be considered in the binding mechanism. According to Edwards and Poulos [1], binding of HF or HCN to CCP results in a network of hydrogen bonds and electrostatic interactions shown schematically in Figure 5. This network is stabilized by interaction of His-52 with the proton of the bound ligand and movement of the positively charged Arg-48 side chain towards the HF ligand by about 2.0 Å and away from the HCN ligand by about 0.3 Å. Rearrangement

of water molecules as observed by Edwards and Poulos [1] at the binding site further stabilizes the hydrogen-bonded network. The binding enthalpies in Table 1 and 2 consist of contributions from these interactions, including the Fe–HX interaction in the binding network. The lack of any enthalpy dependence on temperature indicates that binding occurs without any heat-capacity change which is characteristic of electrostatic and hydrogen-bonding interactions. In comparison, reversible 1:1 dioxygen binding to Fe(II) in horse myoglobin yields a binding enthalpy of $-56.5 \text{ kJ} \cdot \text{mol}^{-1}$ [20] which is close to the observed binding enthalpies of HF and HCN to the Fe in CCP and D82CCP. Thus the enthalpy changes accompanying ligand binding, such as peroxide binding, to CCP are likely to be the same. The binding enthalpies in Tables 1 and 2 are within almost two standard deviations of each other from pH 5.0 to 7.0. In contrast, the binding entropies in Table 1 and 2 exhibit a decrease with increase in pH which accounts for the lower HF-binding constant at neutral pH.

As, with the exception of the movement of Arg-48, the structures of the HF–CCP and HCN–CCP complexes are very similar [1], the thermodynamics of HF binding may be compared with HCN binding despite the number of interactions involved in the binding network. This comparison shows that the binding enthalpy for HCN is significantly more positive, -48.6 ± 1.8 to $-57.2 \text{ kJ} \cdot \text{mol}^{-1}$ for HCN and -67.0 ± 1.9 to -83.8 ± 3.6 for HF, over the pH range studied. If it is assumed that the various contributions to the binding enthalpy are the same for HCN and HF binding, then the more negative values for HF reflect the stronger interaction between the Arg-48 side chain and the ligand in the HF–CCP complex than in the HCN–CCP complex. These results then nicely complement the crystallographic work of Edwards and Poulos [1] which demonstrates movement of the Arg-48 side chain towards the bound fluoride to form a strong interaction, as contrasted with movement of the Arg-48 side chain away from bound HCN. The decrease in entropy on HF binding is almost double that for HCN binding which again reflects a tighter binding network and stronger, more ordered, interactions in the HF–CCP complex.

The overall effect of replacement of the Asn-82 by Asp in D82CCP is weakening of the interaction between the ligand and protein, particularly for fluoride binding. As shown in Figure 4, the fluoride-binding constant for the mutant still follows the same complex pH-dependence as CCP despite the stronger proton affinity of the Asp at position 82 in the mutant. This implies that the amino acid residue at position 82 does not contribute to the observed pH-dependence of the K_b .

As the binding enthalpies of the mutant are at least as negative as those for CCP, the weaker ligand binding to the mutant can be unambiguously attributed to entropic effects. At least two interpretations can be made with regard to the more unfavourable entropic contribution to ligand binding to the D82 mutant. First, the nearly doubled decrease in binding entropy in the mutant could be due to a more ordered tighter network of binding-site interactions existing in the D82CCP–ligand complex compared with the CCP–ligand complex. The stronger proton affinity of Asp-82 in the mutant compared with Asn-82 in CCP would be expected to increase the proton affinity of His-52 and thus its interaction with iron-bound HX, resulting in a more ordered mutant complex. This would be reflected in less of an entropy increase on ligand binding to the mutant than to CCP. However, there is no overwhelming *a priori* reason to expect alterations in proton-donating ability of Asp-82 in D82CCP compared with Asn-82 in CCP to cause a significant increase in ordering in what is already a quite ordered set of local features in the liganded complex. Alternatively, the larger entropy change for D82CCP could result from binding of the ligand to a perturbed enzyme having a greater amount of disorder in its initial unliganded state compared with CCP. Crystallographic studies [3,4] of the CCP–HF complex have demonstrated that the bound fluoride exists in a quite ordered local environment, as judged by well-resolved electron density and unexceptional thermal B factors. Thermal instability of crystals of D82CCP observed in initial attempts to obtain X-ray data for D82CCP (S. F. DeLauder, J. M. Munro, T. L. Poulos, J. C. Williams and F. P. Schwarz, unpublished work) may be taken as circumstantial evidence for a decrease in order in D82CCP which affects the stability of the enzyme molecules or their crystal lattice. Until information about changes in the detailed atomic interactions and degree of ordering of the liganded compared with unliganded binding site can be obtained from X-ray-crystallographic and/or n.m.r.

analysis on the D82 mutant, it is not possible to decide which of these two explanations accounts for the larger decrease in binding entropy of the mutant.

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