Biophysical Chemistry, 43 (1992) 265–277
Elsevier Science Publishers B.V., Amsterdam

BIOCHE 01666

Pressure modulation of cytochrome-to-cytochrome electron-transfer. Models and enzyme reactions

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(Received 22 November 1991; accepted in revised form 19 February 1992)

Abstract

The kinetics of electron-transfer involved in reactions of reduction of 2,6-dichlorophenol indophenol and $Fe(CN)_6^{3-}$ by L-ascorbic acid and reduction of ferric cytochrome *c* by both L-ascorbic acid and reduced hydroxylamine oxidoreductase were studied as a function of three parameters: ionic strength, pressure (1–2000 bar) and temperature (4–20°C) using the high-pressure stopped-flow method. From measurements, the thermodynamic parameters of activation volume ($\Delta V^{\ddagger}$), and, when possible, activation enthalpy and entropy ($\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$) have been calculated. We found, for these four systems, that the pressure has revealed solvation effects involved in electron-transfer. For the reduction of ferric cytochrome *c* by reduced hydroxylamine oxidoreductase (a cytochrome-to-cytochrome electron-transfer), we have not obtained evidence for a conformational change.

Keywords: Hydrostatic pressure; Electron-transfer thermodynamics; cytochrome *c*; hydroxylamine oxidoreductase; Activation volume; Stopped-flow

1. Introduction

The use of pressure as a perturbing physical variable allows the determination of volume changes associated with equilibrium and kinetic constants of reactions [1–4]. Thus, high-pressure biochemical techniques may be powerful tools for investigating enzyme mechanisms [1,5], macromolecular interactions and protein stability [3,6]. In addition, biotechnological applications of the effects of pressure on biochemical materials have opened interesting areas of research in chemical synthesis (drug production) and food processing [7]. Unfortunately, to date interpretation of pressure effects lacks both experimental data and a full theoretical basis. For this reason, a systematic series of investigations of several biochemical reactions is needed. In this context, the present paper is focused upon protein–protein electron-transfer.

In biochemistry, the field of pressure dependence of electron-transfer is limited to the pioneering work of Heremans [8,9] and Swaddle [10], although recent work, devoted to the pressure

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Abbreviations: HAO, hydroxylamine oxidoreductase; DCPIP, 2,6-dichlorophenolindophenol.

dependence of inorganic electron-transfer reactions in solution has demonstrated that pressure can be a very useful physical parameter in mechanistic studies of such processes [11]. Moreover, although even in inorganic chemistry only few results are presently available, they indicate definite trends and shows promising features.

In this paper we report the pressure dependence of some reactions where electron-transfer is involved: two chemical reactions, the reduction of 2,6-dichlorophenolindophenol (DCPIP) and $Fe(CN)_6^{3-}$ by L-ascorbic acid, the reduction of mammalian cytochrome *c* by L-ascorbic acid and the intermolecular electron-transfer from reduced hydroxylamine oxidoreductase to oxidized ferric mammalian cytochrome *c*. The choice of these models was dictated by the time scale of our apparatus. The approach was thermodynamic. Rate constants were determined by stopped-flow as a function of pressure, ionic strength and, when possible, temperature.

This work is an extension of our previous cryo-baro-enzymatic studies on various systems where the thermodynamic activation parameters: $\Delta G^{\ddagger}$ (free enthalpy), $\Delta H^{\ddagger}$ (enthalpy), $\Delta S^{\ddagger}$ (entropy) and $\Delta V^{\ddagger}$ (volume) were determined [4,12–15].

2. Materials and methods

2.1 Enzyme, reagents and solutions

Horse-heart mammalian cytochrome *c* type VI and glucose oxidase were obtained from Sigma, St Louis. Hydroxylamine oxidoreductase (EC 1.7.3.4) from *Nitrosomonas europaea* was prepared according to published procedures [16]. The other chemical compounds were obtained from Merck. Tris-HCl and imidazole-HCl were chosen as buffers, since their H^+ concentration is almost pressure-independent [17,18].

2.2 Kinetic measurements

Kinetic measurements at both atmospheric pressure and high pressure (up to 2000 bar) were made using the stopped-flow equipment developed in the Montpellier laboratory [19,20]. Both devices were interfaced to Aminco DW2 spectrophotometers. Data were stored and analysed as described previously [16]. At least three measurements were performed at each pressure.

(i) The rate of reaction of 2,6-dichlorophenolindophenol with L-ascorbic acid was followed using the dual-wavelength mode of the Aminco: $A_{617\ nm}$ minus $A_{730\ nm}$. The reaction was initiated by mixing equal volumes of DCPIP and ascorbic acid in buffer solution (50 m*M* Tris adjusted to a pH of 8 with HCl). The ionic strength was adjusted by addition of NaCl.

(ii) The reaction of $K_3Fe(CN)_6$ with L-ascorbic acid was started by mixing solutions of both compounds, adjusted to pH 3 with HCl. The reaction was observed in the dual-wavelength mode of the Aminco: $A_{420\ nm}$ minus $A_{475\ nm}$. Sodium chloride was used to vary the ionic strength.

(iii) The rate of reduction of ferric cytochrome *c* was measured by the stopped-flow, mixing solutions of ferric cytochrome *c* in 10 m*M* imidazole buffer, pH 7, with solutions containing L-ascorbic acid in the same buffer system. The equilibrium constant was measured with a Varian 219 spectrophotometer equipped with a thermostated cell holder. Kinetics were recorded in the dual wavelength mode as $A_{550\ nm}$ minus $A_{528\ nm}$. The concentration of ferrous cytochrome *c* was calculated using the extinction coefficient at 550 nm, $\varepsilon_{550} = 27.5\ mM^{-1}\ cm^{-1}$. The ionic strength was varied by addition of NaCl. For the measurement of the rate and equilibrium constants at atmospheric pressure a concentration up to 1 *M* ascorbic acid had to be used. In order to avoid ionic strength effects of this concentration on the reaction, the ionic strength was adjusted to 2 *M* with NaCl for these experiments.

(iv) Electron-transfer from reduced hydroxylamine oxidoreductase (HAO) to oxidized cytochrome *c*. Reduction of hemes of HAO was achieved by its substrate NH_2OH under turnover conditions as follows. In one driving reservoir of the stopped-flow, an aliquot of the concentrated HAO solution was mixed with 20 m*M* Tris buffer pH 7.5, previously deoxygenated by bubbling with oxygen-free argon for 15 min and containing 2

nM glucose oxidase and 60 mM glucose to remove residual oxygen, and NH_2OH. The second driving reservoir of the stopped-flow was filled with cytochrome c solution in the same buffer as that of the first driving reservoir (deoxygenated Tris containing glucose and glucose oxidase). Heme concentrations of ferrous HAO were calculated using $\epsilon_{553} = 27.8\ mM^{-1}\ cm^{-1}$ [16]. Because of some uncertainty in the oligomeric structure of HAO, the concentration of HAO is given per putative active site assuming seven c-type hemes per P460 center [16]. The final concentrations of HAO, NH_2OH and cytochrome c were 1 μM, 1 mM and 6.3 μM respectively. The ionic strength was varied by addition of NaCl. Absorbance changes were recorded against time in the dual mode $A_{418\ nm}$ minus $a_{423\ nm}$. First-order rate constants (k_{obs}) were recorded in a pressure and temperature range of 1 to 1000 bar and 4 to 20°C, respectively.

2.3 Treatment of data

The apparent activation volume ($\Delta V^{\ddagger}$) was calculated from the pressure-dependence of rate constant (k) using the classical equation: $(\partial \ln k/\partial P)_T = -\Delta V^{\ddagger}/RT$ where P is the hydrostatic pressure, T the absolute temperature and R the gas constant (82 ml atm K^{-1}, with 1 atm = 101.3 kPa).

As already suggested for several systems [11,21], a minimum mechanism for electron transfer between A_{Ox} and B_{Red} follows the equation:

$$A_{Ox} + B_{Red} \overset{K_1}{\rightleftharpoons} [A_{Ox} - B_{Red}] \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} [A_{Red} - B_{Ox}] \overset{K_3}{\rightleftharpoons} A_{Red} + B_{Ox}$$

where K_1 and K_3 symbolize rapid equilibria.

By measuring the pseudo-first-order rate constant k_{obs} as a function of B_{Red} concentration, one can obtain K_1 and k_2:

$$k_{obs} = k_2 K_1 [B_{Red}]/(K_1[B_{Red}] + 1) + k_{-2} \qquad (1)$$

It is assumed that k_{-2} is small compared to the other contributions in (eq. 1) and can therefore be neglected. When $[B_{Red}]$ is increased, a plateau for k_{obs} must be reached. Only in the case where this plateau is not reached, i.e. $[B_{Red}] \ll 1/K_1$, k_{obs} is given by

$$k_{obs} = k_+ [B_{Red}], \qquad \text{with } k_+ = K_1 k_2$$

In that case determination of K_1 and k_2 is impossible and only k_+ can be obtained.

3. Results

3.1 Reaction of DCPIP with ascorbic acid

Mixing of DCPIP (0.1 mM) with L-ascorbic acid (20 mM) at 25°C resulted in a pseudo-first-order increase of absorbance at 617 nm. The reaction is thought to proceed by the formation of radicals which then react with each other [22]. An increase of pressure resulted in a slight increase in the rate constant. An increase of Tris concentration slightly increased the negative activation volume of the reaction (see Table 1). The values obtained (−4.3 and −6.6 ml mol^{-1}) are close to the value of −3 ml mol^{-1} (pH 6.5, NaCl = 200 mM) measured by Heremans [23]. Variation of the ionic strength (concentration of NaCl) did not influence the rate constant.

3.2 Reaction of $K_3Fe(CN)_6$ with ascorbic acid

The reaction of $K_3Fe(CN)_6$ with ascorbic acid showed a single exponential decay of the absorbance at 420 nm. The temperature dependence of the reaction was measured at a concen-

Table 1

Values and activation volume for k_{obs} for the reaction of DCPIP with ascorbic acid. [DCPIP] = 50 μM, [Asc] = 10 mM, Tris pH 8. Temperature: 25°C. Values of k_{obs} and $\Delta V^{\ddagger}$ (means ± S.D.) were obtained by computer fitting

Tris concentration (mM)	k_{obs} at 1 bar (s^{-1})	Pressure range (bar)	Activation volume ($\Delta V^{\ddagger}$ in mol mol^{-1})
50	0.21 ± 0.02	1–1500	−4.3 ± 1
400	0.25 ± 0.02	1–1500	−6.6 ± 1

tration of 0.25 mM and 20 mM for $K_3Fe(CN)_6$ and ascorbic acid, respectively, in the temperature range 3.1 to 35°C. From the Arrhenius plot $\Delta H^{\ddagger} = 24.4 \pm 0.8$ kJ mol^{-1} and $\Delta S^{\ddagger} = -156 \pm 2.6$ J K^{-1} mol^{-1} were obtained. The reaction showed a pronounced acceleration with increasing ionic strength. The pressure dependence showed a negative activation volume, i.e. the reaction rate is augmented when the pressure rises. The activation volume did not vary with increasing ionic strength (up to 1 M). In all cases $\Delta V^{\ddagger} = -16 \pm 1.5$ ml mol^{-1}.

3.3 Cytochrome c reduction

The reaction of cytochrome c and L-ascorbic acid was studied under pseudo-first order conditions, where the concentration of L-ascorbic acid greatly exceeded the concentration of cytochrome c. The reaction was first examined at atmospheric pressure. The apparent overall equilibrium constant, determined by a spectrophotometric titration, was: $K_2' = 2.7 \pm 0.3\ 10^5\ M^{-1}$ with: $K_2' = K_1 K_2$; $K_1 = [\text{Cyt } c^{III}\text{-Asc}]/[\text{Cyt } c^{III}]\,[\text{Asc}]$ and $K_2 = [\text{Cyt } c^{II}\text{-Deh.Asc}]/[\text{Cyt } c^{III}\text{-Asc}]$ where Cyt c^{III} is ferric cytochrome c, Cyt c^{II} ferrous cytochrome c, Asc ascorbic acid and Deh. Asc the reduced forms of ascorbic acid.

In Fig 1 the dependence of the observed rate constant (k_{obs}) on the concentration of reductant is shown. The curve can be described by eq. (1). When fitted to a hyperbolic function, values of $K_1 = 0.77 \pm 0.07\ M^{-1}$ and $k_2 = 4.2 \pm 0.4\ s^{-1}$ were obtained (ionic strength, 2 M).

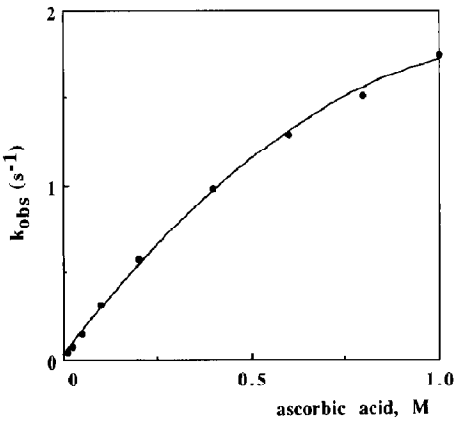

Fig. 1. Dependence of k_{obs} on the reductand concentration for reaction of cytochrome c with ascorbic acid in 10 mM imidazole buffer pH 7 at 25°C. [Cyt c] = 20 μM.

The temperature effect on k_{obs}, determined in the temperature range from 5 to 30°C, led to the activation parameters: $\Delta H^{\ddagger} = 25 \pm 3$ kJ · mol^{-1} and $\Delta S^{\ddagger} = -168 \pm 10$ J mol^{-1} K^{-1}. The reaction showed a significant decrease in reaction rate as the ionic strength was increased (by the addition of NaCl). These results are presented in Fig. 2 together with data for the ionic strength dependence of the reduction of DCPIP and $K_3Fe(CN)_6$ by ascorbic acid.

Table 2 and Fig. 3 illustrate the dependence of k_{obs} on pressure. In all cases the plot of ln k_{obs} versus P was linear and led to a mean negative activation volume, -16.6 ± 1 ml mol^{-1}. This value was independent of the temperature, the concentration of ascorbic acid and the ionic strength.

Table 2

Values and activation volume for k_{obs} for the reaction of cytochrome c with ascorbic acid. [cyt c] = 20 μM in 10 mM imidazole buffer, pH 7. Uncertainty on the k_{obs} values is 5%. Values (means ± S.D.) were obtained by computer fitting

Temperature (°C)	NaCl (mM)	Ascorbic acid (mM)	k_{obs} at 1 bar (s^{-1})	Pressure range (bar)	Activation volume ($\Delta V^{\ddagger}$, in ml mol^{-1})
10	0	2	0.115	0–1900	−16.4 ± 0.8
10	100	2	0.0330	0–1800	−17.3 ± 1.3
25	0	2	0.212	0–1700	−16.0 ± 0.7
25	10	2	0.190	0–1800	−17.7 ± 0.4
25	100	2	0.0627	0–1900	−16.8 ± 0.6
25	0	10	0.960	0–1800	−16.7 ± 0.6

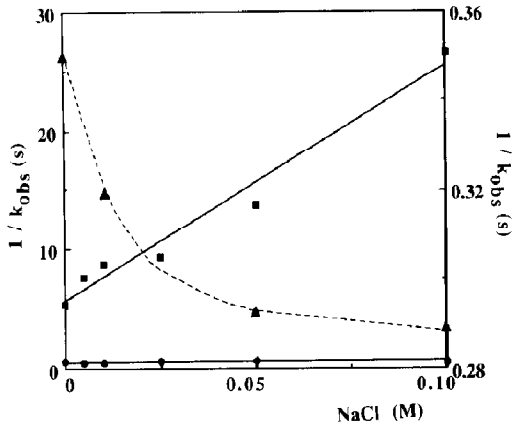

Fig. 2. Reduction of cytochrome c, $K_3Fe(CN)_6$ and DCPIP by ascorbic acid (Asc) at various concentrations of NaCl. The concentrations were respectively: [Cyt c] = 20 μM, [Asc] = 2 mM; [$K_3Fe(CN)_6$] = 250 μM, [Asc] = 20 mM; [DCPIP] = 50 μM, [Asc] = 10 mM. Left hand scale: Cyt (■), DCPIP (●) and right hand scale: $K_3Fe(CN)_6$ (▲). Temperature: 25°C.

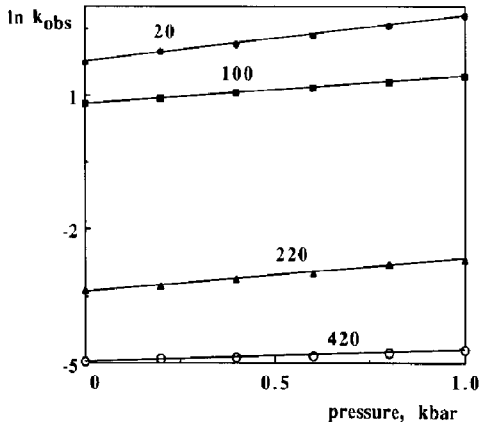

Fig. 4. Dependence of k_{obs} on pressure for the reduction of cytochrome c by reduced HAO at 20°C at various concentrations of NaCl. Final concentrations: [Cyt c] = 3.15 μM, [HAO] = 0.5 μM, [NH_2OH] = 0.5 mM. The final salt concentrations (in mM) are indicated on the figure. Lines were computer fitted.

3.4 *Electron-transfer between reduced HAO and mammalian cytochrome c*

Mammalian cytochrome c can serve as an electron acceptor in the *in vitro* assay of HAO [24,25]. In the present work, then, the reduction of cytochrome c by HAO during turnover of NH_2OH was investigated under various conditions. The kinetics of electron-transfer (as seen by the change in $A_{418\,nm}$ indicating the reduction of mammalian cytochrome c) consisted predominantly of a first-order kinetic (k_{obs}). First, the values of activation volume associated with the reaction (k_{obs}) were studied at 20°C at four values of ionic strength (20 mM Tris pH 7.5 containing 0, 80, 200 and 40 mM NaCl, respectively). Under each condition, k_{obs} remained first-order. Plots of ln k_{obs} versus pressure were linear over the pressure range explored (Fig. 4). Table 3 and Fig. 5 give the dependence of both k_{obs} and $\Delta V^{\ddagger}$ on ionic strength.

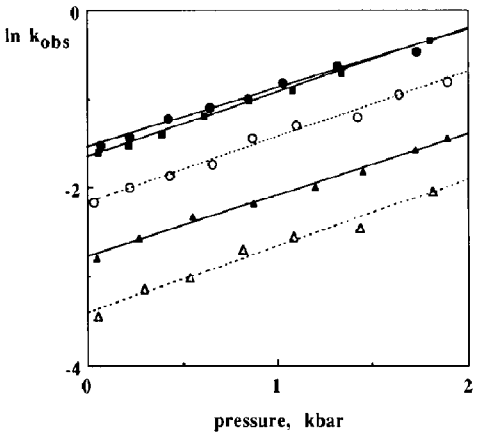

Fig. 3. Dependence of k_{obs} on pressure for the reduction of cytochrome c by L-ascorbic acid at 10 and 25°C at various concentrations of NaCl with [Cyt c] = 20 μM, [Asc] = 2 mM (●), (■) and (▲) at 25°C and 0, 10, 100 mM NaCl, respectively; (○) and (△) at 10°C and 0 and 100 mM NaCl respectively. Lines were computer fitted.

Table 3

Values and activation volume for k_{obs} (at atmospheric pressure) at different ionic strengths for the HAO-cytochrome c reaction. Temperature: 20°C. Conditions as in Fig. 4. Values (means ± S.D.) were obtained by computer fitting

Salt concentration (mM)	k_{obs} (s^{-1})	$\Delta V^{\ddagger}$ (ml mol^{-1})
20	5.7 ± 0.1	−24.3 ± 0.4
100	2 ± 0.05	−22.5 ± 1
220	0.033 ± 0.001	−17.1 ± 0.7
420	0.007 ± 0.0001	−5.6 ± 0.3

The values of $\Delta V^{\ddagger}$ obtained in 20 mM Tris were virtually independent of temperature: -22.7 ± 2, -20.6 ± 2 and -24.3 ± 0.4 ml mol^{-1} at 4, 10 and 20°C, respectively. Within experimental error the values of $\Delta H^{\ddagger}$ were not affected by change in temperature, pressure or ionic strength. Activation energies at pH 7.5 were 42 ± 2 kJ mol^{-1} and 42.5 ± 4 kJ mol^{-1} in 20 mM Tris and 20 mM Tris containing 200 mM NaCl, respectively. These data are close to the value obtained in previously determinations in 20 mM phosphate buffer, pH 7.5 containing 0.1 M NaCl (50 ± 1 kJ mol^{-1} [25]).

3.5 Effect of pressure on the rate of reduction of hydroxylamine oxidoreductase by hydroxylamine

The kinetics of reduction of *c*-heme of the enzyme by its substrate were previously determined as a function of pressure at one ionic strength [14]. The dependence of $\Delta V^{\ddagger}$ on ionic strength is reported here. Experiments consisted of mixing enzyme contained in one syringe of the stopped-flow (concentration before mixing: 4.5 μM) with NH_2OH contained in the second syringe (concentration before mixing: 40 mM). The buffer was 20 mM Tris, pH 7.5, containing NaCl when necessary. Due to the very high velocity of the reaction followed at 552 nm, the temperature

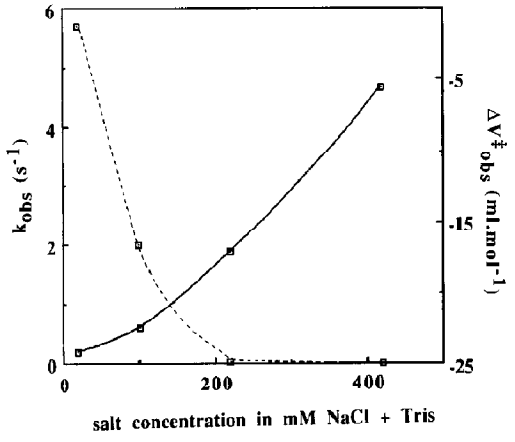

Fig. 5. Effect of salt concentration on reaction rate (left hand scale, - - -) and on activation volume (right hand scale, -) on the reaction of cytochrome *c* by reduced HAO. Conditions as in Fig. 4.

Table 4

Values and activation volume for k_1 for the reduction of HAO by NH_2OH at different salt concentrations (concentrations of HAO and NH_2OH were 2.25 μM and 20 μM, respectively). Temperature: 3.5°C. Values (means ± S.D.) were obtained by computer fitting

Salt concentration (mM)	k_1 (s^{-1})	$\Delta V^{\ddagger}$ (ml mol^{-1})
20	14.0 ± 0.1	−0.7 ± 0.3
100	14.1 ± 0.1	−1.8 ± 0.4
420	13.1 ± 0.5	−1 ± 1

was fixed at 3.5°C. The value of k_{obs} (14 s^{-1} in NaCl-free buffer at 3.5°C) corresponds nicely with published values [14]. The kinetics consisted of two first-order phases, the fast phase k_1 (which was used for calculations) represented 90% of the change in absorbance of the reduced *c*-hemes (see ref 14). Data are in Table 4. The previously determined value of $\Delta V^{\ddagger}$ in 100 mM Tris, pH 7.5, was -1.8 ± 0.5 ml mol^{-1} at 3.5°C [14].

4. Discussion

Investigation of electron-transfer between HAO and cytochrome *c* was chosen for the ease of assay using the high pressure stopped-flow method. Furthermore, the reaction can be used as a model for the physiological reaction of HAO with cytochrome *c*554 [25], which will be investigated later.

Due to the complex nature of the interpretation of the activation volume parameter in biochemical electron-transfer reactions [9], we first investigated simple chemical reactions as models. In order to facilitate comparisons we first studied the chemical reduction of DCPIP by ascorbic acid, the same reductant as in the first biochemical example studied in this work (ascorbic acid–cytochrome *c*). In the second chemical example (ascorbic acid–$K_3Fe(CN)_6$) we chose an iron complex as being somewhat analogous to cytochrome *c*, which also contains Fe as its redox center. The reaction of cytochrome *c* and ascorbic acid was also studied, since it may reflect on the biochemical electron-transfer (HAO to cy-

tochrome *c*). The choice of ascorbic acid as the reductant was imposed by the fact that reduction with available iron complexes (like $K_4Fe(CN)_6$) is too fast to be monitored using stopped-flow methods. The comparison between the two couples (ascorbic acid–$K_3Fe(CN)_6$ and ascorbic acid–cytochrome *c*) permits the analysis of the role of the polypeptide chain of the protein.

In this work we have tried to progressively complicate the experimental systems in order to allow interpretation of the action of pressure on protein–protein electron-transfer.

4.1 DCPIP–ascorbic acid

The reduction of DCPIP by ascorbic acid occurs in a biochemical pH range. At pH 8 the reaction proceeds via the mono deprotonated ascorbic acid and a mono deprotonated species of DCPIP [22]. The reaction involves the initial formation of radicals of both substrates which then decay to give final products. It is assumed that the formation of the radicals is the ratelimiting step. The reaction rate is not influenced by ionic strength in the range of 10 to 200 mM (see Fig. 1). This is in keeping with the absence of electrostatic interactions in reactants. The reaction shows a small negative activation volume (-4.3 ± 1 ml mol^{-1} in 50 m*M* buffer), which slightly increases when the buffer concentration is raised from 50 to 400 mM (-6.6 ± 1 ml mol^{-1} in 400 m*M* buffer). However, given the small value for the observed activation volumes and the rather large experimental error (which is more than 20% in this case and is always at least in the order of magnitude of 1 to 2 ml mol^{-1} regardless of the absolute value of the measured volume changes) we cannot interpret these changes. For this reaction which involved only radical species, the small negative activation volume found experimentally can be attributed to solvent reorganization, as already concluded in other systems [8].

4.2 $K_3Fe(CN)_6$–ascorbic acid

The reaction of ferricyanide and ascorbic acid was measured far from physiological values (pH 3) because the reaction was too fast to be observed at pH 7. At this acidic value, the ascorbic acid should be completely protonated but it is not known which form of ascorbic acid is the reactive species. The reduction step is the rate-determining step in this reaction. Taking into account the overall concentration of ascorbic acid, the calculated second order rate constant is 160 M^{-1} s^{-1}. This value is in the same order of magnitude than rate constants for the reduction of cytochrome *c* by ascorbic acid (see Table 5).

Table 5

Rate constants for the reaction of cytochrome *c* with ascorbic acid

T (°C)	pH	Buffer concentration (m*M*)	*k* [M^{-1} s^{-1}]	Reference
20	7.4	Phosphate, 10	300	40
20	7.4	Phosphate, 67	60	40
20	7.4	Phosphate, 300	25	40
25	7.3	Hepes, 10	50	27
25	7.0	Cacodylate, 1	240	41
25	8.0	Tris, 10	1300	42
25	8.0	Tris, 100	450	42
25	7.0	Imidazole, 10	200	This work

The reaction is strongly accelerated by increasing ionic strength. In the ionic strength range of 0.01 to 2 *M* the rate constant changed from 2.86 s^{-1} to 8.49 s^{-1}. This is due to the fact that ions of the same sign of charge are involved in the reaction.

The observed activation volume of -16 ± 1.5 ml mol^{-1} and the other activation parameters (small $\Delta H^{\ddagger}$ and negative $\Delta S^{\ddagger}$) support an outer-sphere mechanism for this reaction [11,26].

4.3 Cytochrome c–ascorbic acid

4.3.1 Kinetic and thermodynamic parameters at atmospheric pressure

The following minimum reaction scheme can be suggested for the reaction between cytochrome *c* and ascorbic acid:

$$\text{Cyt } c^{III} + \text{Asc} \overset{K_1}{\rightleftharpoons} \text{Cyt } c^{III}\text{I}^{II} \cdots \text{Asc} \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} \text{Cyt } c^{II} \cdots \text{Deh.asc} \overset{K_3}{\rightleftharpoons} \text{Cyt } c^{II} + \text{Deh.Asc}$$

where Cyt c^{III} is ferric cytochrome c, Cyt c^{II} ferrous cytochrome c, Asc ascorbic acid and Deh.asc the reduced forms of ascorbic acid. K_1 and K_3 represent fast equilibria, in which collision complexes are formed ([Cyt $c^{III} \cdots$ Asc] and [Cyt $c^{II} \cdots$ Deh.asc], respectively). This minimum reaction scheme was also given by Heremans [8] and Van Eldick [11] for similar electron-transfer reactions.

The reduction step k_2 is the rate-limiting step, where a conformational change of cytochrome c could be involved. From both the measurement of the equilibrium constant $K_2'(K_2' = K_1K_2)$ as well as the fitting of the kinetic data to eq. (1), which gave the values of K_1 and k_2, we calculated $K_2 = 3.5 \pm 0.4 \cdot 10^5$ and $k_2 = 4.2 \pm 0.4$ s^{-1}. The value of $k_{-2} = k_2/K_2$ is very small ($1.5 \cdot 10^{-5}$ s^{-1}). This small value can therefore be neglected in eq. (1). The observed rate constant is then described by:

$$k_{obs} = k_2K_1[\text{Asc}]/1 + K_1[\text{Asc}] \qquad (2)$$

(K_3 has not been determined since no significant spectral change was observed between the complex [Cyt $c^{II} \cdots$ Deh.asc] and Cyt c^{II}).

In the literature the reaction of cytochrome c with ascorbic acid was studied at ascorbic acid concentration as high as 250 mM [27]. Only in one publication a saturation effect at much lower concentrations of ascorbic acid was observed [28]. This effect was shown to be due to changes in the ionic strength [27]. In our investigation we tried to rule out the possibility of an ionic strength effect by adjusting the ionic strength with NaCl (1:1 electrolyte like Na Asc, the ascorbic acid should be totally present in the mono-protonated form at pH 7). We observed a linear dependence of the rate constant on the concentration of reductant up to 250 mM as observed by Williams [27]. Only at higher concentrations a deviation from linearity was observed which led to the calculation of K_1.

In order to compare our rate constant with literature (see Table 5) a simplified reaction scheme had to be used.

$$\text{Cyt } c^{III} + \text{Asc} \rightarrow \text{Cyt } c^{II} + \text{Deh.Asc}$$

With this reaction scheme k_{obs} is described by

$$k_{obs} = k[\text{Asc}]$$

where [Asc] is the total concentration of ascorbic acid. We obtained a value of 200 M^{-1} s^{-1} which is in good agreement with the data presented in Table 5. The reaction proceeds via the double deprotonated species Asc^{2-} [26]. In order to get a rate constant with the real concentration of the reactive species (Asc^{2-}) the concentration of this species had to be taken instead of the overall concentration of ascorbic acid. This led to a value of $3\ 10^6\ M^{-1}$ s^{-1} which is in the same order of magnitude as the values obtained at other ionic strengths by Williams ($8 \cdot 10^5\ M^{-1}$ s^{-1}) [27] and Al-Ayash ($3.1 \cdot 10^5\ M^{-1}$ s^{-1}) [26]. These values are in the same range as values for other reactions of cytochrome c proceeding via an outer-sphere mechanism [29—31].

Electron-transfer reactions involving metal complexes can generally be classified proceeding via inner-sphere or outer-sphere mechanisms depending on whether a ligand exchange is involved in the reaction or not [11]. It has been pointed out that the reaction of cytochrome c with ascorbic acid proceeds via an outer-sphere mechanism [26] which is characterized by a small activation enthalpy and a negative activation entropy. The data presented here confirm this statement (see Table 6).

A decrease of the observed reaction rate (k_{obs}) from 0.212 to 0.0627 s^{-1} with increasing ionic strength (NaCl concentration in the range of 0 to 100 mM, see Table 2) is similar to that observed previously [27,32,33]. This classic effect of ionic strength can be interpreted in terms of the involvement of electrostatic interactions [34].

4.3.2 Pressure effect

Differentiation of equation (2) with respect to the pressure yields:

$$\frac{\partial \ln k_{obs}}{\partial p} = -\frac{\Delta V^{\ddagger}_{obs}}{RT}$$

$$= -\frac{\Delta V_2^{\ddagger}}{RT} - \frac{1}{1 + K_1[\text{Asc}]}\frac{\Delta V_1}{RT} \qquad (3)$$

Table 6

Comparative activation parameters for the reduction of different cytochromes

System	$\Delta H^{\ddagger}$ (kJ mol^{-1})	$\Delta S^{\ddagger}$ (J K^{-1} mol^{-1})	Ref.
Cytochrome c_1 (III)–asc. acid	29.7	−163	25
H.h. cytochrome *c* (III)–asc. acid	11.5	−92	25
Cytochrome c_{551} (III)–asc. acid	1.5	−178	25
H.h. cytochrome *c* (VI)–asc. acid	25	−168	This work
H.h cytochrome *c* (III)–FeII (EDTA)	21	−88	29

H.h. = horse heart.

At low ascorbic acid concentrations $K_1[\mathrm{Asc}] \ll 1$. The above equation is then simplified:

$$\Delta V^{\ddagger}_{obs} = \Delta V^{\ddagger}_2 + \Delta V_1$$

At high ascorbic acid concentrations the contribution of ΔV_1 decreases, thus allowing the evaluation of the two terms ΔV_1 and $\Delta V^{\ddagger}_2$. Unfortunately, in the present experiment, the ascorbic acid concentration was limited by technical problems to the range of 2 to 10 m*M*, a range where $\Delta V^{\ddagger}_{obs}$ did not vary. The measurements at ascorbic acid concentrations higher than 10 m*M* failed because diffusion can occur at the level of the mixing chamber of the high-pressure stopped-flow device since the two driving syringes are not closed by valves [19,20]. At ascorbic acid concentrations above 10 m*M*, then concentration differs by a factor of at least 2000 so that a slight exchange occurring between the two solutions and diffusion at the mixing chamber level can rapidly lead to complete reduction of the cytochrome *c*.

For the reaction of ascorbic acid with cytochrome *c* the deprotonation of the mono-protonated form of ascorbic acid to the totally deprotonated form has to be taken into account when discussing volume changes [8,26]. The observed activation volume, $\Delta V^{\ddagger}_{obs}$ thus can be described by

$$\Delta V^{\ddagger}_{obs} = \Delta V^{\ddagger}_{reaction} + \Delta V_{asc}[\mathrm{H^+}/K_{AH} + \mathrm{H^+}]$$

where ΔV_{asc} is the reaction volume for the deprotonation of ascorbic acid and $\Delta V^{\ddagger}_{reaction}$ the real activation volume of the reaction. The reaction volume of ascorbic acid dissociation has been estimated from other thermodynamic parameters to be −12 ml mol^{-1} [8]. In our experiments $[\mathrm{H^+}/K_{AH}+\mathrm{H^+}] = 1$, so ΔV_{asc} contributes a factor of 1 to the observed volume changes. The ΔV_{asc} term has to be added to eq. (3). Subtraction of this value leads to −4.6 ml mol^{-1} for the electron-transfer reaction. Heremans [8] calculated the total theoretical value for this activation volume taking into account first, the electrostatic work to bring the reactants together, second, the activation volume for the solvent reorganization, and third, a reaction volume related to a compressibility factor of the solvent. He obtained a value of −1 ml mol^{-1}. The term for the solvent reorganization was calculated to be −4.7 ml mol^{-1}. Heremans and van Eldik calculated this term also for other electron-transfer reactions proceeding via an outer-sphere mechanism, where the solvent reorganization term is always in the order of −2.5 to −7.5 ml mol^{-1}.

The activation volume determined in this work (−16.6 ± 1 ml mol^{-1}) is smaller than that determined by Heremans (−23 ml mol^{-1}) [8]. This difference may be due to differences in the experimental conditions of the Heremans experiments: Tris was used as buffer and Na_2SO_4 used to adjust the ionic strength whereas, here, the buffer and salt were imidazole and NaCl, respectively. Correcting the obtained activation volumes for the contribution of ascorbic acid leads to a value of −11 ml mol^{-1} for the Heremans experiment whereas here a value of −5.6 ml mol^{-1} is obtained. This value is somewhat nearer to the theoretically determined value of −1 ml mol^{-1}.

Within experimental errors, no dependence of volume change on ionic strength (range 10 to 200 m*M*) or temperature (10 to 25°C) was observed (see Table 2).

The reaction of cytochrome *c* and ascorbic acid proceeds via an outer-sphere mechanism where a negative activation volume is predicted (whereas positive reaction volumes are expected for inner-sphere mechanisms) [35]. However, Heremans [8] showed that the sign of the activa-

Table 7

Comparative activation parameters for the reduction of cytochrome *c* and $K_3Fe(CN)_6$ (see text for conditions)

System	$\Delta H^{\ddagger}$ (kJ mol^{-1})	$\Delta S^{\ddagger}$ (J K^{-1} mol^{-1})	$\Delta V^{\ddagger}$ (ml mol^{-1})
H.h. cyt.c (VI)– asc.acid	25	−168	−16.6
$K_3Fe(CN_6)$– asc.acid	24.4	−156	−16

H.h. = horse heart.

tion volume is a doubtful criterion to distinguish between outer- and inner-sphere mechanisms since the measured activation volume includes both the reaction volume of the complex formation (term ΔV_1) and the reaction volume related to the electrostatic interaction (term $\Delta V_2^{\ddagger}$). He calculated a ΔV_1 value for the complex formation of 1.5 ml mol^{-1} [8]. Depending on the contribution of K_1 and k_2 for k_{obs}, the $\Delta V_{obs}^{\ddagger}$ will reflect either ΔV_1 or $\Delta V_2^{\ddagger}$.

All activation parameters for the reductions of $K_3Fe(CN)_6$ and cytochrome *c* by ascorbic acid are shown in Table 7. These values are very close together and may indicate that in these two reactions the rate-determining step (reflected by the measured activation parameters) is the same. The protein shell does not seem to influence the reaction. A similar behaviour is observed for the reaction of cytochrome *c* with Fe(II) EDTA [29].

4.4 Electron-transfer from NH_2OH-reduced HAO to cytochrome c

This reaction, commonly used as a test of the activity of HAO, is a model reaction for the periplasmic physiological electron-transfer from reduced HAO to tetraheme cytochrome *c*554 in *Nitrosomonas europaea*. The isoelectric points of HAO (p*I* 4.7) and cytochrome *c* (p*I* 10.8) are complementary. At atmospheric pressure, the strong dependence on ionic strength previously observed in the rate of reaction was interpreted as typical for a simple electrostatic interaction between the two proteins [25]. Present data (see Fig. 5) extend this observation to include a dependence of $\Delta V^{\ddagger}$ on salt concentration. We note that in the range of ionic strength explored (20–420 m*M*), ln k_{obs} varies linearly with pressure over the entire range of pressure used (1 to 1000 bar), indicating that no pressure-induced denaturation nor variation of rate-determining step occurred [14]. We must note that, in spite of its multiheme structure, between 1 bar and 1.5 kbar, the HAO did not undergo detectable structural changes in high pressure polyacrylamide gel electrophoresis [36].

An apparent activation volume, $\Delta V_{obs}^{\ddagger}$, can be considered as the sum of several components [4]:

$$\Delta V_{obs}^{\ddagger} = \Delta V_{chem}^{\ddagger} + \Delta V_{conf}^{\ddagger} + \Delta V_{solv}^{\ddagger}$$

involving chemical, conformational and solvation terms, respectively. The solvation term includes also the solvation term of the electron-transfer process itself. In the present experiments, the chemical term is related to the electron-transfer itself.

At low ionic strength, the fact that the activation volume has a negative value ($\Delta V_{obs}^{\ddagger} = -24.3 \pm 0.4$ ml mol^{-1} at 20 m*M* salt concentration) could suggest the involvement of an outersphere process. Since this value is relatively close to the value described above for the reduction reaction of cytochrome *c* (−16.6 ml mol^{-1}) and to values published elsewhere [8,9], it seems to indicate that at this ionic strength, the contributions of both $\Delta V_{conf}^{\ddagger}$ and $\Delta V_{solv}^{\ddagger}$ are small.

4.5 Solvation effect

The absolute value of $\Delta V_{obs}^{\ddagger}$ decreases as the ionic strength increases (Table 3). Low and Somero [37,38] found a similar correlation between the effect of salts on the rates of enzyme reactions and their effects on the activation volumes of the reactions. Based on the V_{max} of kinase and various dehydrogenases, they observed that salts (of the Hofmeister series) that increase $\Delta V^{\ddagger}$ are invariably accompanied by a decrease of the reaction rate and *vice versa*. In the experiments described, a linear relationship between rate and activation volume at different salt concentrations indicated that salts probably modify volume changes by affecting the degree to

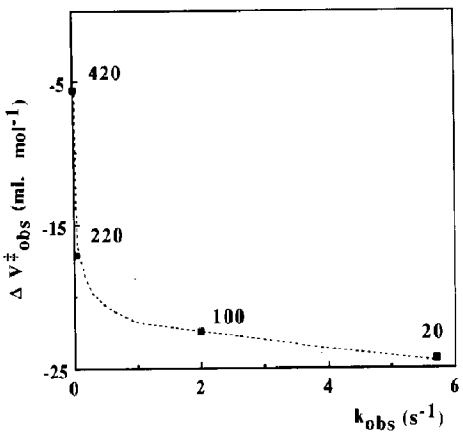

Fig. 6. Relationship between reaction rate and activation volume at different salt concentrations (in mM on the figure) for the reaction of cytochrome c with reduced HAO. Conditions as in Fig. 4.

which water organizes around amino acid side chains and peptide linkages located near the protein–water interface [38].

For the electron-transfer from NH_2OH-reduced HAO to cytochrome c, phenomena are different as compared with Low and Somero conclusions. Although $\Delta V^{\ddagger}_{obs}$ is dependent on the ionic strength, there is not a linear correlation between reaction rate and activation volume at different salt concentrations (see Fig. 6). Due both to the complexity of the HAO molecule (Mr $\approx$ 200 000 with an α_3 subunit composition which contains 21 c-hemes and three P-460 moieties [39]) and of the processes involved, it is, at this stage, impossible to propose the details of the kinetic analysis of the phenomenon of the sort that can be given for the reduction of cytochrome c with ascorbic acid. However we offer one hypothesis. With increasing ionic strength, the outer-sphere process term $\Delta V^{\ddagger}_{chem}$ is compensated by the solvation term $\Delta V^{\ddagger}_{sol}$ having an opposite positive sign. The non-linearity observed in Fig. 6 could then be due to an increasing salting-out effect associated with increasing ionic strengths as well as the solvation term $\Delta V^{\ddagger}_{sol}$ discussed above. From a practical point of view, it means that under pressure, the velocity of an electron transfer rate is faster at low ionic strength than at high ionic strength and faster than at 1 bar. We must point out that at high ionic strength, where the complex between the two proteins is weak, the $\Delta V^{\ddagger}$ (-5.6 ml. mol^{-1} at 3.5°C, see Table 3) is a macroscopic thermodynamic response value close to the expected contribution of solvent reorganization calculated at room temperature [8,15].

With the present model reaction, we have not obtained evidence for a conformational change during the process (as indicated by the $\Delta V^{\ddagger}_{conf}$ term). However, evidence for a conformational change was previously observed using high pressure methods in the presence of organic cosolvent for a more specific reaction where intramolecular electron-transfer takes place between the P-460-containing site and a series of c-hemes in HAO [14]. On the other hand, in another series of experiments at atmospheric pressure, it has been postulated that HAO exists in two or more conformational states having different binding and kinetic properties [25].

We note that although it is not yet possible to assign a biochemical counterpart to a given $\Delta V^{\ddagger}_{obs}$, the present experiments provide example of solvation effects involved in electron-transfer for both model and enzyme reactions. The study of the pressure dependence of the physiological reduction of cytochrome c554 by hydroxylamine oxidoreductase, where electron-transfer governs the reaction [25], is in progress.

5. Summary and conclusions

In this work we have examined four different systems of increasing reaction complexity.

In the first, pure chemical reaction of DCPIP with ascorbic acid a small negative activation volume was found which was interpretated in terms of solvation changes. As this reaction proceeds via radicals, a direct comparison with the three other systems which include reactions with metal complexes can not be made.

In the case of the reactions of K_3FeCN_6 and cytochrome c with ascorbic acid, the same activation parameters were observed indicating that the same step is rate-limiting in both reactions and

that both proceed via an outer-sphere complex mechanism. This implies that the protein chain of cytochrome *c* seems not be involved in the rate-determining step. The changes in reaction rate with increasing ionic strength are classically interpreted by the electrostatic interactions.

In the fourth reaction between reduced HAO and cytochrome *c* both reaction rate and activation volume were strongly influenced by rising ionic strength. This is interpreted in terms of changes in solvation of the reacting compounds and a possible salting out effect with increasing ionic strength. Conformational changes which were previously observed for a more specific reaction could not be detected in this reaction. Because of the complex nature of HAO a detailed interpretation of the activation volume is not possible at present.

However, a clear interpretation of activation volume for reactions involving biochemical compounds is still a problem, as the overall reaction volumes are not known. As pointed out by Marcus and Sutin similar difficulties exist for other activation parameters [43].

Acknowledgements

We should like to acknowledge stimulating discussions with Dr J. Frank (Delft University). We thank the Compagnie Rhone-Poulenc, Department of Silicones, for the gift of Rhodorsil oil and Mr J.L. Saldana for technical assistance. This work was supported by grants from La Fondation pour la recherche Médicale Française, the Direction des Recherches, Etudes et Techniques (91/1044J), the PROCOPE program and INT-8413813 of the U.S. National Science Foundation.

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