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Activation thermodynamics of the binding of carbon monoxide to horseradish peroxidase

Role of pressure, temperature and solvent

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The kinetics at 423 nm of the binding of carbon monoxide to ferrous horseradish peroxidase were studied as a function of three parameters: pressure (1–1200 bar), temperature (34 to −20 °C) and solvent (water, 40% ethylene glycol, 50% methanol) using a high-pressure stopped-flow apparatus. By using transition state theory the thermodynamic quantities $\Delta V^{\ddagger}$, $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ were determined under these different experimental conditions and were found to be greatly modulated by the physico-chemical parameters of the media. The results suggest that the macroscopic thermodynamic response is mainly controlled by the solvent. By adjusting two variables (among T, P, solvent), it is possible either to amplify or to cancel out the effect of the third.

1. Introduction

An enzyme reaction pathway involves several elementary steps, each of which is described by two kinetic constants. By studying these in terms of transition state theory [1], it is possible to obtain information about the thermodynamic parameters pertaining to the activated states that allow for the transition between successive complexes (see, for example, ref. 1).

With enzymes, the most commonly exploited thermodynamic variables are temperature and pressure, such studies leading to the thermodynamic activation parameters: $\Delta G^{\ddagger}$ (variation in standard free enthalpy), $\Delta H^{\ddagger}$ (enthalpy), $\Delta S^{\ddagger}$ (entropy) and $\Delta V^{\ddagger}$ (volume) (for reviews, see refs. 2–7, and for recent works, see refs. 8–13). A problem with these parameters is that, when taken separately, their interpretation is not easy [2]. However, as pointed out by Low and Somero [14,15], one can gain information by perturbing the system, e.g., by varying the physicochemical properties of the medium (solvent, salt, pH, etc.).

Here, we studied the properties of the elementary rate constants describing the binding of carbon monoxide to horseradish peroxidase under various conditions of temperature (T), pressure (P) and solvent (for a recent paper on this system, see ref. 16). To increase the precision of the data, we covered wide ranges of temperature and pressure by employing specially constructed, rapid-reaction equipment [17,18]. By using cryosolvents (40%, v/v, ethylene glycol; 50%, v/v, methanol) we were immediately able to extend the temperature range to subzero conditions [6,19] and to perturb the system.

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Abbreviations: HRP, horseradish peroxidase; DMSO, dimethyl sulfoxide; EGOH, ethylene glycol; MeOH, methanol.

This work is an extension of our previous thermodynamic studies on horseradish peroxidase with peroxides as substrates [20] and on certain other systems [21–25]. It adds to several studies on the effects of pressure on the binding of oxygen and/or CO to heme proteins [26–29]. Although a quantitative interpretation of the results remains premature, it is only by collecting data on various systems that we might obtain a description of the activation parameters with respect to solvent effects. Interestingly, we show that by adjusting any two of the three variables (T, P and solvent), it is possible either to amplify or to cancel out the effect of the third.

2. Materials and methods

2.1. Enzyme reagents and solutions

Horseradish peroxidase (HRP; EC 1.11.1.7, type VI, from Sigma, St. Louis) consisted almost exclusively of the C isoenzyme [30]. Concentrations were calculated using $\epsilon_{403} = 102.3\ mM^{-1}$ [31]. Solutions of CO were prepared by diluting a saturated solution in water taken to be 1.0 mM at 20 °C under atmospheric pressure. Tris-HCl was chosen as buffer, since its H^+ concentration is almost pressure-independent [32]. The paH (activity of H^+ in hydro-organic buffer) was estimated according to procedures published elsewhere [33].

To reduce HRP, a small aliquot of a concentrated protein solution was mixed with buffer solution deoxygenated by bubbling with oxygen-free argon for 15 min. The protein was then reduced by addition of solid sodium dithionite. This solution was transferred anaerobically using a syringe to one of the two driving reservoirs of the stopped-flow apparatus (see below) previously deoxygenated by bubbling with argon for 30 min and treatment with sodium dithionite. The second driving reservoir was filled with CO solution previously deoxygenated with dithionite.

2.2. Kinetic measurements

Kinetic measurements at atmospheric pressure were made with a stopped-flow apparatus adapted to cryoenzymic conditions and constructed in this laboratory [34]. Experiments at high pressures and low temperatures were performed using a high-pressure device recently developed in this laboratory [17,18]. Both devices, which have a 'dead time' of mixing of less than 5 ms, were connected to an Aminco DW2 spectrophotometer. The data were absorbance changes recorded vs. time in the dual-wavelength mode of the spectrophotometer: specifically, the difference in absorbance at 423 and 500 nm, where the difference between the values of the absorbance of the HRP-CO compound is greatest [35]. Data were stored in a Datalab DL901 transient recorder connected to an Apple II microcomputer and analysed using the Kinfit program [36]. Rapid-scan absorption spectra were recorded under atmospheric pressures using the stopped-flow apparatus [34] adapted to a Union Giken RA 415-RA 401 (Osaka, Japan) fast-response spectrophotometer.

3. Results and discussion

3.1. Pathway of the CO binding process: solvent and temperature effects

The general 'induced-fit' theory implicitly implies that the binding of the ligand CO to the protein is a two-step process: formation of a collision complex (rapid attainment of equilibrium) followed by an isomerization step:

$$E + CO \overset{K_1}{\rightleftharpoons} E \cdot CO \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E^* \cdot CO$$

Spectroscopic measurements of the formation of $E^* \cdot CO$ provide the first-order kinetic constant $k_{obs} = k_{-2} + k_2[CO]/K_1 + [CO]$, where K_1 is the dissociation constant. When [CO] is increased a plateau for k_{obs} must be reached.

The kinetics (at 423 nm) were studied at pH 7.5 (or paH) in either aqueous solution (4–34 °C), 50% methanol (20 to −20 °C), or 40% ethylene glycol (20 to −10 °C). Under all conditions, the resulting curves were clearly first order and the rate constants obtained (k_{obs}) were linearly dependent on the concentration of CO (up to 0.5

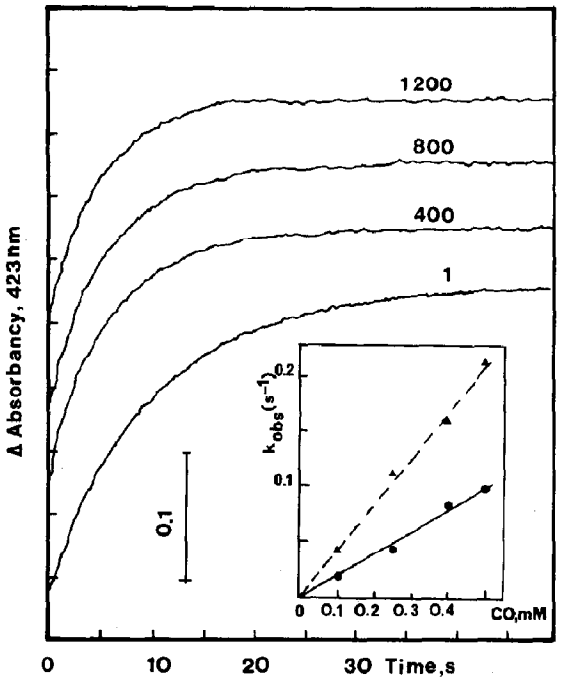

Fig. 1. Kinetics of formation of the (HRP-CO) complex in 0.2 M Tris, paH 7.5 in 40% (v/v) EGOH at different pressures as indicated. Temperature: −10 °C. For the sake of clarity, curves have been offset on the absorbance ordinate. Inset, dependence of k_{obs} on the concentration of CO, at −10 °C, in 40% EGOH. 1 bar (●), 1200 bar (▲).

mM) (fig. 1). Rapid-scan spectra (not shown) indicated that, under hydro-organic conditions, the absorption spectra had the same characteristic shape as when determined in the absence of organic solvent.

The linearity of k_{obs} as a function of CO concentration (fig. 1) means that K_1 and k_2 remain too high for the maximum CO concentration allowed (0.5 mM). For other systems, such as myosin ATPase [37] or the HRP-peroxide system [20], the use of an organic cosolvent and/or subzero temperatures permitted us to obtain saturation curves. We did not succeed with the HRP-CO system, probably because k_2 and/or K_1 remain too high. Finally, we obtained only the second-order constant $k_+ = k_2/K_1$ (the slope in $M^{-1}\ s^{-1}$), the values of which are summarized in table 1. In addition, under the same conditions, extrapolation

Table 1

Values and activation volume for k_+ under different experimental conditions

Values (means ± S.D.) were obtained by computer fitting of ln k_+ as a function of pressure.

	Temperature (°C)	k_+ ($\times 10^{-3}$) ($M^{-1}\ s^{-1}$)	$\Delta V^{\ddagger}$ (ml mol^{-1})
Water	34	6.2 ±0.6	−23.6 ±1
	20	2.7 ±0.4	−23.7 ±1
	4.2	1.0 ±0.1	−26.9 ±1
40% EGOH	20	2.87±0.02	−6.98±0.02
	0.5	0.57±0.01	−10.5 ±0.6
	−10	0.2 ±0.01	−14.6 ±0.8
50% methanol	20	5.6 ±0.1	−9.4 ±0.2
	10.3	3.4 ±0.2	−6 ±0.2
	4.2	1.5 ±0.1	−5.5 ±0.2
	0	1.3 ±0.1	−5.2 ±1
	−9.8	0.6 ±0.1	−2.3 ±0.3
	−20	0.25±0.1	−1.6 ±0.4

of k_{obs} to zero concentration gave its value to be close to zero, which implies a very small k_{-2}.

The main feature concerning these results is the small solvent effect on k_+ which, for example, at 20 °C, varies by less than a factor of 2 for the three solvents. In comparison, in the hydroxylamine oxidoreductase-CO reaction, the presence of 40% ethylene glycol increased the velocity of the binding rate constant by a factor of 2.5–3 [25]. Further, we observed an increase in to a factor of 10 for the binding of peroxides to HRP [20].

Table 2

Thermodynamic parameters for k_+ under different experimental conditions

Values (means ± S.D.) were obtained by computer fitting.

Thermodynamic parameters	Pressure (bar)	Medium		
		Water	40% EGOH	50% MeOH
$\Delta G_+^{\ddagger}$ (20 °C)	1	53.1±0.1	52 ±1	51±2
(kJ mol^{-1})	1200	50 ±2	51.6±0.1	50±1
$\Delta H_+^{\ddagger}$	1	41.7±0.1	55 ±1	47±2
(kJ mol^{-1})	1200	34 ±2	45.7±0.1	53±1
$\Delta S_+^{\ddagger}$	1	−37.2±0.2	8 ±4	−13±8
(J $mol^{-1}\ K^{-1}$)	1200	−52 ±6	−20.2±0.5	11±4

The question remains as to the thermodynamical significance of this constant k_+. The equilibrium constant K_1 is dealt with by using the Van't Hoff equation $\Delta G_1 = -RT \ln(K_1)$ for the complete transformation of 1 mol E + 1 mol CO at a concentration of 1 M to give 1 mol complex (E-CO) (standard conditions). The rate constant k_2 is interpreted in terms of the activated state theory of Eyring et al. [1] which postulates that there is a transition state $(E \cdot CO)^{\ddagger}$ between (E · CO) and (E* · CO) the properties of which are described by $\Delta G_2^{\ddagger} = -RT \ln(hk_2/kT)$ for the transformation of 1 mol (E · CO) to $(E \cdot CO)^{\ddagger}$ (h and k denote the Planck and Boltzmann constants, respectively).

The $\Delta G_+^{\ddagger}$ associated with the overall constant k_+ is equal to $\Delta G_2^{\ddagger} - \Delta G_1 = -RT \ln(hk_+/kT)$. This is symbolized by the energy diagram shown in fig. 2. Finally, $\Delta G_+^{\ddagger}$ describes the thermodynamic properties of the activated complex $(E \cdot CO)^{\ddagger}$ with reference to the ground state (E + CO), independently of the properties of the collision complex (E · CO). We can have a situation where the last complex is very sensitive to the conditions of the medium (T, P or solvent) but where $(E \cdot CO)^{\ddagger}$ is not: K_1 and k_2 fluctuate simultaneously but k_2/K_1 remains relatively constant. Such a situation has been observed with the myosin ATPase [37] but not for the HRP-peroxides reaction [20].

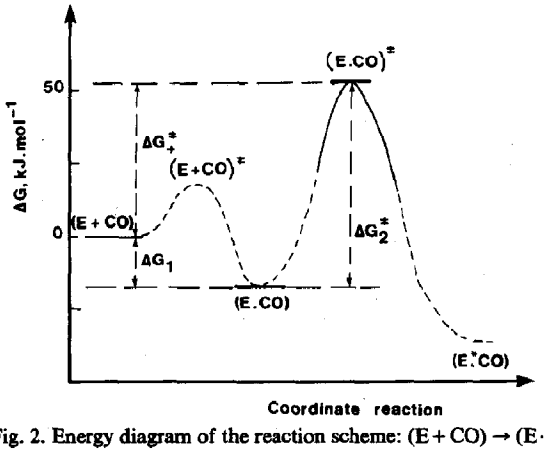

Fig. 2. Energy diagram of the reaction scheme: (E + CO) → (E · CO) → (E* · CO), E representing HRP. Values are those in water at 20 °C and 1 bar. Dashed curves represent the unknown values.

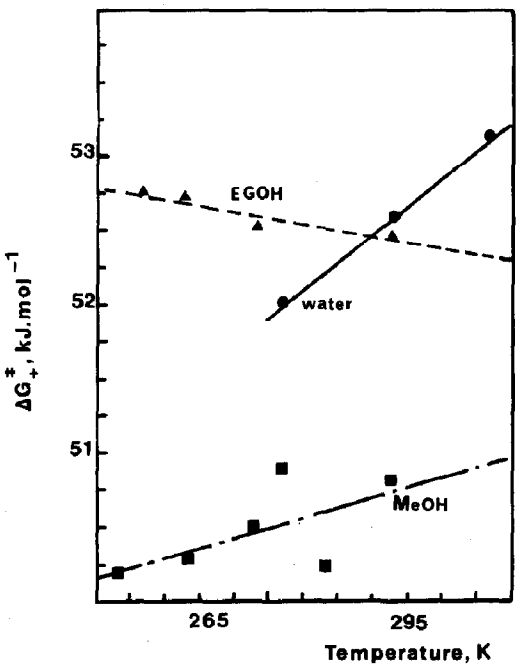

Fig. 3. Plot of $\Delta G_+^{\ddagger}$ vs. T for k_+ in water (●), 40% EGOH (▲) and 50% methanol (■) at atmospheric pressure. For details, see text.

The variations of $\Delta G_+^{\ddagger}$ as a function of temperature at atmospheric pressure and in different solvents are demonstrated in fig. 3. The relationship $\Delta G_+^{\ddagger} = \Delta H_+^{\ddagger} - T\Delta S_+^{\ddagger}$ gives $\Delta H_+^{\ddagger}$ and $\Delta S_+^{\ddagger}$ the values of which are summarized in table 2.

3.2. Pressure effect

The influence of pressure on the binding of CO was studied in the three solvents and at different temperatures. Under each condition, k_{obs} remained first order and linear as a function of [CO], from which k_+ is obtained. According to transition-state theory, the pressure dependence of k_+ is given by: $(\delta \ln k_+/\delta P)_T = -\Delta V_+^{\ddagger}/RT$ where $\Delta V_+^{\ddagger} = \Delta V_2^{\ddagger} - \Delta V_1$.

A plot of ln k_+ vs. pressure should be linear. This is the case under all conditions investigated (in the pressure range 1–1200 bar) and the slopes of the lines give $\Delta V_+^{\ddagger}$, assuming $R = 82$ cm^3 atm

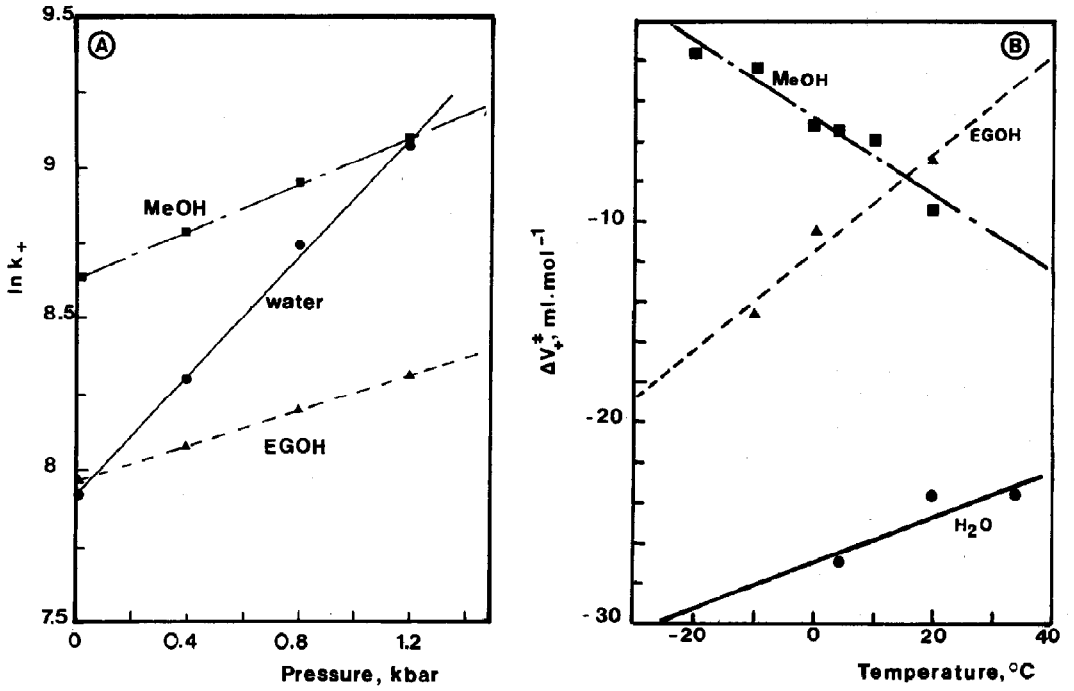

Fig. 4. (A) Dependence of k_+ on pressure and (B) dependence of $\Delta V_+^{\ddagger}$ for k_+ on temperature. Water (●), 40% EGOH (▲) and 50% methanol (■). Temperature: 20 °C. The lines were computer fitted.

Table 3

Temperature coefficients of activation volume and pressure coefficients of activation entropy and enthalpy for k_+ in different media Values (means ± S.D.) were obtained by computer fitting.

Coefficients	Medium		
	Water	40% EGOH	50% MeOH
$(\delta\Delta V^{\ddagger}/\delta T)_P$ (ml mol^{-1} K^{-1})	0.11 ± 0.05	0.24 ± 0.05	−0.19 ± 0.02
$(\delta\Delta S^{\ddagger}/\delta P)_T$ (ml mol^{-1} K^{-1})	−0.10 ± 0.05	−0.24 ± 0.02	0.21 ± 0.01
$(\delta\Delta H^{\ddagger}/\delta P)_T$ (ml mol^{-1})	−55 ±10	−78 ± 5	52 ±2
$\Delta V^{\ddagger} - T(\delta\Delta V^{\ddagger}/\delta T)_P$ (ml mol^{-1})			
Temperature (°C)			
34	−58 ±15		
20	−56 ±15	−78 ±15	47 ±6
4.2	−58 ±13		
0		−77 ±13	47 ±5
−9.8			48 ±5
−10		−79 ±13	
−20			48 ±5

K^{-1} mol^{-1} with 1 atm = 1.013×10^5 Pa (see fig. 4A). The values obtained are listed in table 1.

The linearities observed in fig. 4A for the three solvents deserve further comment. First, curvature of the plots can be a consequence of a change of the rate-limiting step at high pressures, as observed, e.g., in the fumarase reaction [13]; the linearity obtained validates the reaction pathway proposed. Second, interpretation of the data does not necessitate reference to Kramers' equations [38] which are required when the ranges of temperature and pressure are large compared to those used here [29]. This shows that variations in the viscosity under our conditions remain slight and in this case transition-state theory appears as an approximation of that of Kramers as developed by Butz et al. [13]. Third, it means that the compressibility $\Delta\kappa = (\delta\Delta V_+^\ddagger/\delta P)_T$ remains small in extent. ($\ll 10^{-3}$ ml mol^{-1} bar^{-1}) [39]. In addition, variations in $\Delta V_+^\ddagger$ as a function of temperature are linear as shown in fig. 4B.

The $\Delta V_+^\ddagger$ observed is the sum of several components: the binding of CO, then a subsequent conformational change occurring in the protein, and the associated reorganization of the solvation shell. In fact, these events are interconnected and thus difficult to analyse independently. However, at this stage we point out that the large variations observed in $\Delta V_+^\ddagger$ under different conditions suggest that solvent reorganization is the predominant factor [14,15] and that this drives the response of the system.

3.3. Thermodynamic considerations

Finally, we obtained values for $\Delta H^\ddagger$, $\Delta S^\ddagger$ and $\Delta V^\ddagger$ which describe the properties of the activated state $(HRP\text{-}CO)^\ddagger$ in relation to the ground state (HRP + CO) as a function of the three parameters P, T and solvent composition. Table 3 lists the calculated values for derivatives of $\Delta H_+^\ddagger$, $\Delta S_+^\ddagger$ and $\Delta V_+^\ddagger$ with P and T as variables. These values give rise to an accurate fit to Maxwell's relationships: $(\delta\Delta V_+^\ddagger/\delta T)_P = -(\delta\Delta S_+^\ddagger/\delta P)_T$ and $(\delta\Delta H_+^\ddagger/\delta P)_T = \Delta V_+^\ddagger - T(\delta\Delta V_+^\ddagger/\delta T)_P$. A similar behaviour has been observed for other biochemical reactions [25,28]; this merely shows that the results are internally consistent: the correlation is a mathematical consequence of the definitions of thermodynamic functions as recently pointed out by Hamann [40].

An interesting point is that all these parameters vary widely as a function of the experimental conditions. For example, alterations in temperature cause significant changes in $\Delta V_+^\ddagger$: -24 ml mol^{-1} in water at 34°C and -1.6 ml mol^{-1} at -20°C in 50% methanol. $\Delta V_+^\ddagger$ increases with decreasing temperature when the solvent is water or 40% ethylene glycol but decreases in 50% methanol. Also, $\Delta S_+^\ddagger$ is very sensitive to the conditions, in particular to pressure (see table 2).

When $\Delta G_+^\ddagger$ is plotted as a function of P at various temperatures in 40% ethylene glycol (fig. 5A) and in 50% methanol (fig. 5B), straight lines are obtained which appear to converge towards a zone where $(\delta\Delta G_+^\ddagger/\delta T)_P = \Delta S_+^\ddagger \simeq 0$ for 350 ± 50

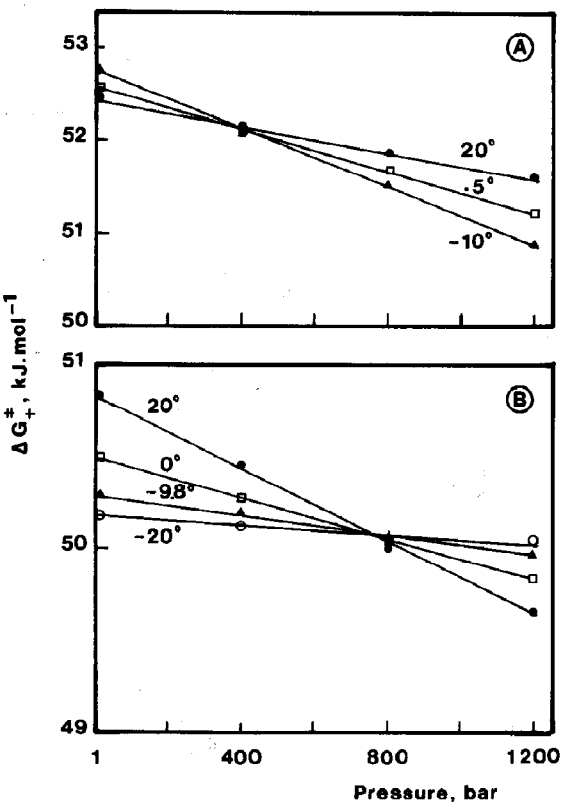

Fig. 5. Dependence of $\Delta G_+^\ddagger$ on pressure at selected temperatures as indicated. (A) In 40% EGOH and (B) in 50% methanol. The lines were computer fitted.

bar in 40% ethylene glycol and 750 ± 50 bar in 50% methanol. At these points, $\Delta S_+^\ddagger$ for the process is close to zero. For another system [25], a stationary point was observed for $\Delta H^\ddagger$ where the temperature was without effect: at higher pressure, the temperature effect was inverted and decreasing temperature led to an increase in the rate of reaction.

At $-30\,^\circ$C, in 50% methanol, the pressure effect is slight ($\Delta V_+^\ddagger \simeq 0$) and at $0\,^\circ$C and 300 bar, compared with pure water, methanol has no effect on k_+ as with 40% ethylene glycol at $20\,^\circ$C and 100 bar.

These results show that by adjusting two variables, one can minimize the effect of the third.

We must point out that despite the fact that the nature of the observed phenomena always remains the same, with identical spectral variation and rather similar values of the rate constants, certain thermodynamic values such as $\Delta V^\ddagger$ vary dramatically or even change sign, for instance, $\Delta S^\ddagger$ or $\delta \Delta V^\ddagger / \delta T$. As already suggested by various authors [3,7,14,21,41,42], it is probably an indication that the thermodynamic macroscopic response of these systems is mainly controlled by solvent reorganization. We observe little change in $\Delta H_+^\ddagger$ and $\Delta S_+^\ddagger$ by varying the solvent (table 2) but it seems that there is a compensatory effect: when $\Delta H_+^\ddagger$ increases the term $-T\Delta S_+^\ddagger$ decreases. The amplitudes of these variations are too small for one to verify quantitatively the theory on the compensation, however, this qualitative aspect also helps one to explain the predominant part of the solvation in the phenomenon under study [43]. Further investigations necessitate a physical model for the protein-solvent system. A recent preliminary approach [44] has attempted to draw a parallel between the structural transitions observed in proteins and those observed in macroscopic gels as a function of the physico-chemical parameters of the medium. A compilation of results such as those presented here might test this hypothesis.

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