EFFECT OF TEMPERATURE ON AC, WAVES OF LYSOZYME

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SUMMARY

Disulphide bonds of lysozyme were reduced on dme *and* hme *electrodes. The heterogeneous kinetic process yielded apparent activation energies that involves adsorption contributions. However, Vlcek plots show a only one mechanism of reduction at the 5-50 °C interval. The chemisorption of this protein was made evident by the evolution of the recorder at a selected phase angle of $\phi = \pi/2$ rad. At 50 °C two electroactive disulphide bonds of lysozyme were reduced simultaneously.*

INTRODUCTION

Few proteins are as well known as lysozyme. Shortly after its primary structure was determined[1,2], it became the first enzyme for which the three-dimensional structure was elucidated[3]. On the basis of structural considerations, protein chemists have divided lysozyme into four segments. A deep crevice containing the active site divides the molecule into two halves. On one side of the crevice is the ß-structured second segment, and on the other side are the two chain-terminal α-helical segments. The third segment, with a regular α-helical element, joins these two halves. All the cystine residues are in α-helical

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segments[3]. Lysozyme has four disulphide bridges, but only the two near the surface, solvent accesible bridges(6-127 and 30-115 residues) are reduced by sodium sulphite in urea[4]. Also, from catalytic polarographic waves of lysozyme in Co(II)-ammonia buffer solutions, only four half-cystine residues are accesible to the mercury electrode [5].

The reduction of the disulphide bonds depends on protonation , adsorption and structural rearrangement steps [6-8]. The adsorbed proteins on mercury interfaces are denaturalized [9] during a long potentiostatic reduction. The adsorption of globular proteins at mercury solution interfaces provides evidence of unfolded molecular conformation. Lysozyme has a high polar character and many authors suggest that structural changes occur several seconds after incorporation into the interfaces[10]. Lysozyme shows strong conformational changes with the pH and concentration [11,12]. In the present work the effect of temperature on the first harmonic alternating polarograms of lysozyme is studied. The study is made in buffered moderated acid media as in these circumstances the protonation of disulphide bonds is relatively rapid [13]. The objective is to obtain structural and electrochemical information of lysozyme in the medium/mercury interfaces from ac polarograms.

EXPERIMENTAL

The polarograms were recorded by Metrohm E-506 polarograph with a three electrode system, using a thermostated cell. Potentials were referred to the Ag/Ag Cl/K Cl_{sat} electrode. The capillary used had a mercury flow rate of m = 2.61 ± 0.01 mg. s^{-1} at -1.0 V., when it was inmersed in 0.1 M K Cl solution. The drop time was mechanically fixed at

a value of 0.6 s. The pH was measured with a PHM 62 Radiometer pHmeter. Temperature was maintained constan using a cryostat-thermostat Heto with a precision of ± 0.1 °K precisions using a cryostat-thermostat Heto. Briton-Robinson buffer solutions were used. The ionic strength was controlled by addition of potasium chloride [14]. Tricristallyzed, diallyzed and liophillyzed lysozyme was supplied by Sigma S.A.

RESULTS

The lysozyme is reduced and reoxidized on the *hme* by means of a heterogeneous process where the protonations steps are coupled with monoelectronic transfers.

The height of the ac peaks increases with the temperature (Fig. 1). From Arrhenius $\ln i_p$ *vs* 1/T(K) plots, an apparent activation energy of 7 Kcal.mol^{-1} is obtained at the potential of the peak.

From the current values at different temperatures, mantaining the potential constant, we can draw Arrhenius plots. The apparent activation energies, Q, obtained by these methods(Fig.2), obeys the equation:

$$Q = Q^{\circ} - n\alpha_Q F E \qquad (1)$$

as some authors postulate for other electrochemical processes[15]. The $n\alpha_Q$ values obtained from this equation are near to 2 . This number coincides with the number of electrons needed in each electronic transfer step of the reduction of two disulphide bridges, so with the overall number of electrons needed in the reduction of each

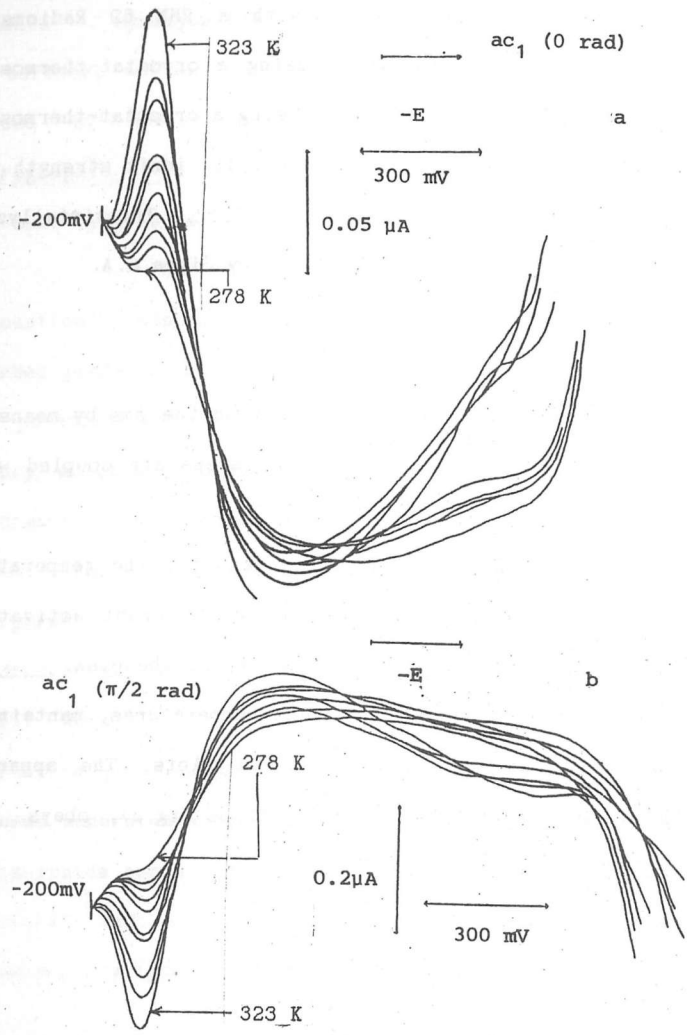

Fig.1.- ac₁ Polarographyc waves of lysozyme at 5,10,15,20,25,30,35,40,45,50 ºC.

pH = 5.0; I = 0.5; t = 0.6 s; m = 2.61 mg s^{-1}; ΔE = 10 mV; 0.2% of lysozyme.

a) Phase sensitive angle, ϕ = 0 rad.

b) Phase sensitive angle, ϕ = π/2 rad.

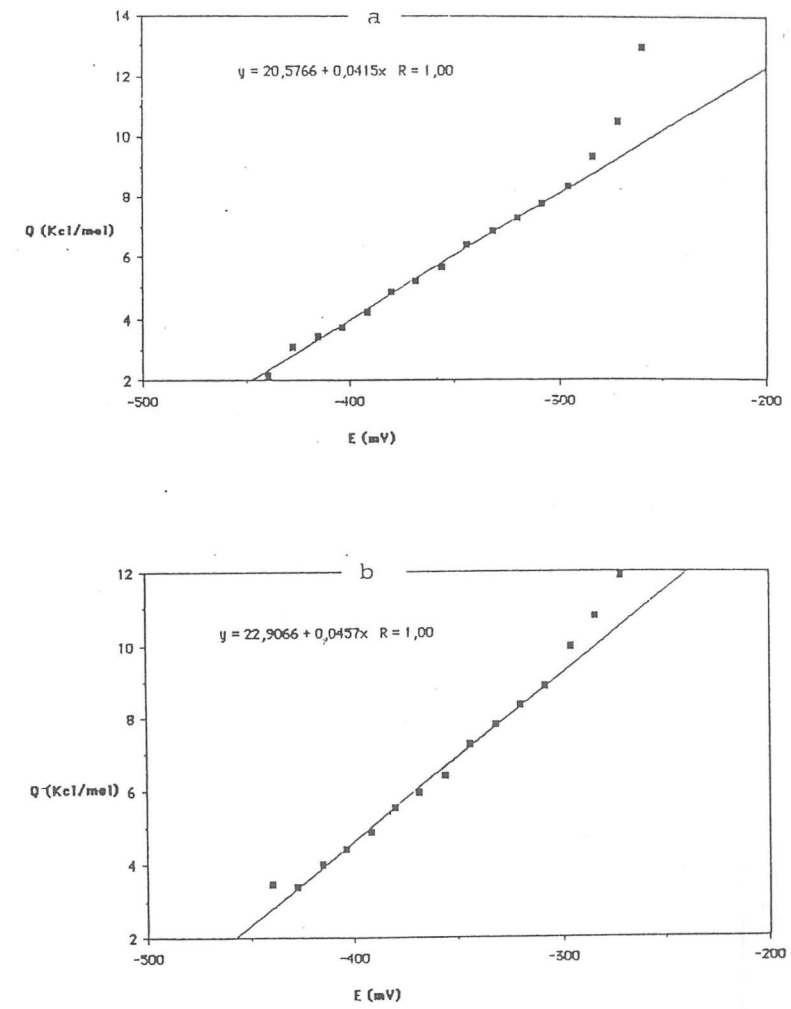

Fig.2.- Apparent activation energies at different potentials.

pH = 5.0; I = 0.5; t = 0.6 s; m = 2.61 mg s^{-1}; Δ = 10 mV; 0.2% of lysozyme.

a) ac_1 Polarography at ϕ = 0 rad.

b) ac_1 Polarography at ϕ = π/2 rad.

electroactive disulphide bridge , but differs on the nα values obtained from the polarographic curves (Table I).

The contribution of the adsorption phenomena is very important. When the temperature increases, the faradaic process is favoured sice the protein is chemically adsorbed on dme by the -S-S- disulphide bonds (7). Then the ac_1 ($\phi = \pi/2$ rad) (Fig. 1b) peak increases. Also at more cathodic potentials appears a litle new faradayc process (T > 298 K) that coincides with the denaturation of the lysozyme and with the change in the slope of the semilogarithmic plots of figure 3.

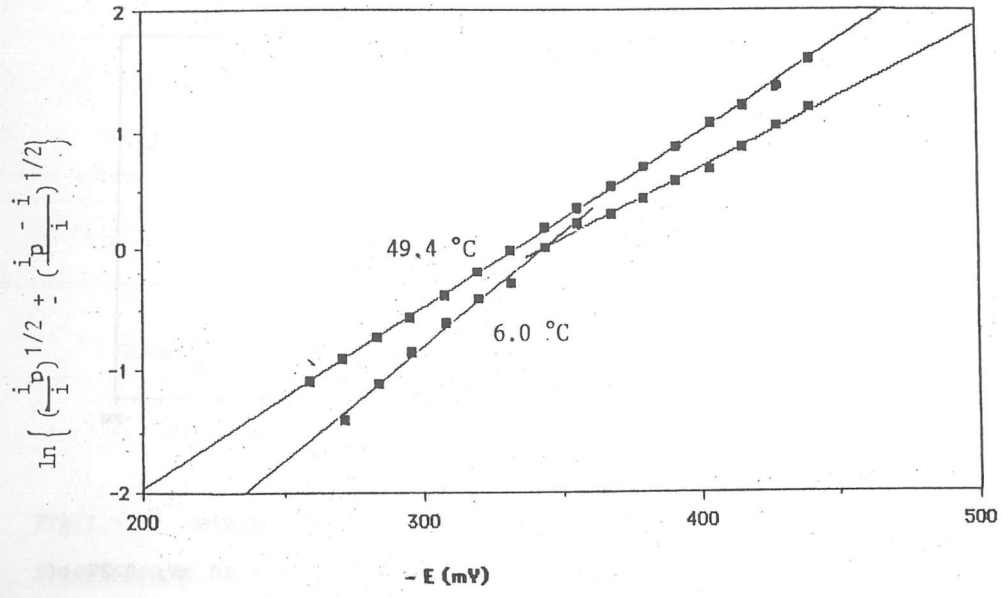

Fig. 3.- Smith plots of ac_1 waves at different temperatures

In the same experimental conditions that Fig. 2a).

This strong temperature effect on the waves is shown in the Smith semilogarthmics plots[15]. When the temperature is varied from 5 to 50 °C, the initial two slopes evolve to only one value (Fig.3). The nα experimental parameter changes in this intervale of temperatures from 0.5 to $n\alpha \simeq 0.8$. Then, in all temperature intervals the first monoelectronic transfer after the first protonation occurs at the same potentials as the second monoelectronic transfer. We can explain the evolution of nα -values from 0.5 to 0.8 by the conformational changes and desorption of the protein with the temperature. This means that when the temperatura increases, the steric difficulties disappear and the two electroactive disulphide bonds are equivalent on the *dme*.

The variation of i_p and E_p with temperature (table I) in the disulphide reduction waves is a characteristic of the structure and agregational state changes of the protein. Generally, as in the case of the commercial insulins and another globular proteins, the Arrhenius treatment gives non- linear plots, because the adsorption and structural changes affect to the electrodic process [6]. In the case of lysozyme, Vlcek plots [17] yield only one straight line for each potential tested (Fig.4). The semilogarithmic plots of $\ln\{(i_p/i)^{1/2} \pm (i_p - i/i)^{1/2}\}$ *vs* $10^3\ T^{-1}$ are valid because the superimposed potential applied is small (ΔE = 10 mv), and the ac_1 waves adquieres the form of the first derivate of sigmoidal polarograms.

Chemical Arrhenius plots of lysozyme have been studied at different pH's [18]: a sharp break was noted at physiological pH and temperature, reflecting the temperature-sensitive molecular rearrangement. The thermal conformational transition of lysozyme, with

Table I.- Effect of temperature on peak potentials and nα parameters. pH = 5.0; Ionic strength, I = 0.5; drop time, t = 0.6 s; mercury flow, m = 2.61 mg s^{-1}; amplitude of superimposed alternating potential, ΔE = 10 mV; Phase sensitive angles, ϕ = 0 rad and ϕ = π/2 rad. The nα parameter was calculated from semilogarytmic Smith plots.

T(ºC)	- E_p(mV)		nα	
	ϕ=o	ϕ=π/2 rad	ϕ=0	ϕ=π/2 rad
6.0	344	352	0.57	0.52
10.8	347	349	0.53	0.48
15.6	345	346	0.54	0.56
21.0	342	351	0.63	0.54
25.4	341	345	0.63	0.56
31.0	337	342	0.65	0.65
35.4	337	345	0.68	0.59
40.8	336	333	0.70	0.67
44.6	336	336	0.74	0.72
49.4	333	331	0.82	0.76

the temperature is clearly demostrated at pH=5 by ^{13}CNMR spectroscopy. The observed pH dependence of the temperature-induced rearrangements [18] thus suggests a possible involvement of some carboxilyc groups in the transition. The heterogeneous reduction of the

disulphide bridges of lysozyme on mercury electrodes do not clearly show the thermal conformational transition since they are chemically adsorbed. Only the irregular evolution of the electrochemical nα parameters with the temperature denotes structural rearrangements.

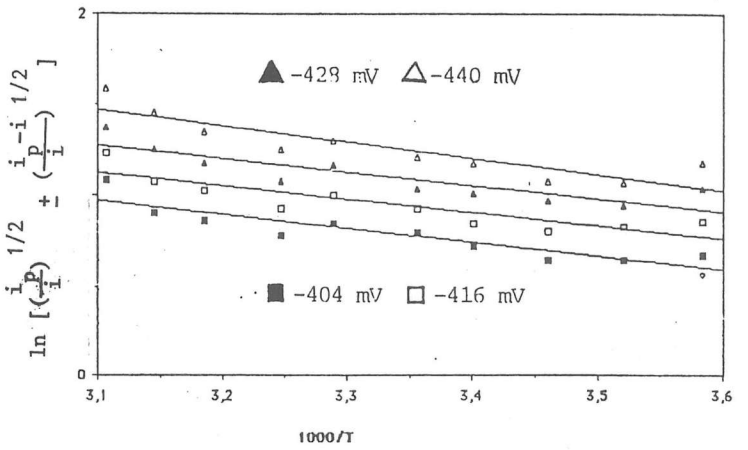

Fig.4.- Adapted Vlecek plots to ac, waves at different potentials. In the same experimetal conditions as Fig. 2 a) and Fig. 3 a).

DISCUSSION

For an irreversible electrode process, the Vlcek[19] recommendation for tast polarographic waves consist in determining an activation energy, Q', referred to as an arbitrary constant potential, E', and the theoretical equation:

$$\ln \{i/(i_l - i)\} = \ln (A') - (Q' - 1/2\ Q_D)/RT - n\alpha_Q \cdot F\ (E_{1/2} - E')/RT, \quad (2)$$

is useful. Whichever of the quantities A' and Q' are determined it is still necessary to know Q_D. This quantity may be readily determinated from the temperature dependence of ln i_l. The slope of ln i_l versus 1/T graph being 1/2 (Q_D/R) if the electrode process is controlled by diffusion. This slope is useful for mechanistic assumptions in first order processes. Obviously, therefore, no absolute physical meaning must be attached to Q' values. A non-linear dependence is an indication that the reaction mechanism changes with temperature and the process might take place by means of more than one path.

On the other hand, the therm "transfer coefficients" suggest that a fraction of the energy is changed by the species involved in electron transfer processes. But is difficult to see why part of the energy is transferred during a monoelectronic transference in the electrochemical reaction. All the cases that we have tested are explained by an extended Devanathan model[20,21]. Independent of the nature of the process, we can consider the electron transfer very rapid. The chemical or physical coupled processes cause retardation in the rate of the process.

From this assumption the transfer coefficient α coincides with the simmetry factor $\beta = 1/2$ for a monoelectronic transference, acquiring a probabilistic significance. The transferred electron cannot be distinguished between the oxidized and reducted forms. So, there are two equally probable possibilities for each electron transferred.

From a phenomenologycal experiment we can determine the $n\alpha$ parameter. This experimental value is related to $\beta=1/2$ by simple equations that include the contributions of structural rearrangements on the interface, ω',

$$n\alpha = (j-1)+\beta+\omega' \quad (3)$$

and other parameter, j, that depend on the nature of the reaction mechanism (21) (is the monoelectronic transference energetically determining of the wave position in the potential axes). From semilogarithmic treatment of sigmoidal curves (i.e. dc polarography, normal pulse polarography, K_1 and K_2 Kalousek polarographic methods), we can determine the $n\alpha$ parameter. And in recorders having a peak shape (ac_1 and dp polarography,), we can extend this treatment considering the peak shape as the derivative of a sigmoidal curve. Only two conditions are needed: a) The electrochemical process are of first order with respect to electroactive species and b) The perturbation to the applied potential slope are small, i.e. , amplitude pulse in dp polarography $\Delta E \leq 10$ mV or the amplitude of superposed ac potential $\Delta E \leq 10$ mV. So, in both cases, $E_p \simeq E_{1/2}$, and the $n\alpha$ differs or coincides to that obtained from dc polarography depending on interfacial behaviour of the sustancies from their respective perturbations. Therefore, in these

circumstances, Smith plots for ac; waves (and dp) are equivalent to semilogarithmic Tomes plots. When the graphycal plots do not give one single straight line we can infer that there are superimposed electrochemical processes or changes in the mechanism, or other contributions as adsorption which are not considered in the equations. In all the cases, Tomes, Smith, Vlcek and Arrhenius plots, and their modifications are of usefuless in understanding the kynetic nature of involved electrochemical processes.

Weaver has discussed the temperature dependence of the apparent transfer coefficients in terms of both enthalpic and entropic contributions to Gibbs energy of activation[22]. The actual behaviour of temperature dependence of Tafel slopes for any irreversible electrochemical reaction is $b = 2.3RT/[(\beta_H + \beta_S T)]F$ [15]

Then, we can write a general equation :

$$n\alpha = n\alpha_H + n\alpha_S\, T \qquad (4)$$

wherein $n\alpha$ is the experimental measured value, $n\alpha_H$ the hentalpic contribution and $n\alpha_S$ the entropic contribution. The first contribution is independent of the temperature . The second contribution implies structural changes in the interface. Therefore, from equations (3) and (4):

$$n\alpha_H \simeq (j-1) + \beta \qquad (5)$$

and

$$n\alpha_S\, T \simeq \omega'. \qquad (6)$$

When only one wave is detected in acid media for the reduction of disulphide bonds ,

$$-S-S- + 2H^+ + 2e^- \leftrightarrow 2(-SH)\ ,$$

the first monoelectron transfer (j=1) controls the potential needed for reducing the disulphide bonds, since the preceeding chemical steps are kynetically rate determining. So, from equation (5), $n\alpha_H = 0.5$ is obtained, and $\omega' = n\alpha - 0.5$. The entropic contribution depends on two phenomena: the self structural changes of the protein and the desorption of the protein on the mercury electrode when the temperature increases. The value of the ω' parameter is related to the number of molecules of water displaced on *dme* by each electroactive disulphide bond[21]. However the equations (5) and (6) are only of cualitative usefunless since the calculated ω' parameter includes also electrostatic, enthalpic and very important experimental error.

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ELECTROCHEMICAL CLEAVAGE OF CHLORO DERIVATIVES OF THE BENZYLOXYCARBONYL GROUP

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ABSTRACT

The cathodic cleavage of 4-chlorobenzyloxycarbonyl and 2,4-dichlorobenzyloxycarbonyl groups from the urethane derivatives of morpholine, at a vitreous carbon cathode in N,N-dimethylformamide is reported.

It is shown by cyclic voltammetry that the derivatives are reduced in the region - 2.4 to - 2.6 V *vs* SCE; further reduction peaks are observed at more negative potentials.

Controlled potential electrolyses were carried out at a potential just after the first reduction peak and free amine was identified in moderated yields. Hence, the first process is associated with the cleavage of the modified Z groups, but undesired reactions also occur leading to cleavage in other sites of the molecule.

INTRODUCTION

In peptide synthesis is common to introduce modifications in the aminoacid/peptide molecules in the form of protecting groups, in order to prevent unwanted reactions away from the site where the change is desired.

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