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TEMPERATURE DEPENDENCE OF INTRAMOLECULAR ELECTRON TRANSFER AS A PROBE FOR PREDENATURATIONAL CHANGES IN LYSOZYME

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Intramolecular electron transfer in hen egg-white lysozyme between tryptophan and tyrosine units was investigated by means of pulse radiolysis in the temperature range 288–333 K. An Arrhenius plot for the kinetics of this process shows a sharp break at ~ 303 K (30°C) compatible with the trend noted earlier (cf P. Jolles, *et al. BBA*, **491**, 354, (1977)) on the Arrhenius plot for kinetics of bacterial substrate digestion by lysozyme. The departure from linearity of the Arrhenius plot for intramolecular electron transfer is interpreted in terms of local intralobe fluctuations of the native structure of lysozyme. It is suggested that such an approach can be useful for probing predenaturational changes in proteins.

KEY WORDS: Lysozyme, intramolecular electron transfer, tryptophyl and tyrosyl radicals, pulse radiolysis, activation parameters, predenaturational changes.

INTRODUCTION

Long-range intramolecular electron transfer reactions involving tryptophan, tyrosine, methionine, and cysteine residues, as well as metal centers have been observed in numerous peptides and proteins.[1-6] The factors that control the rates of such processes include the donor-acceptor separation distance, thermodynamic driving force ($\Delta E^{\circ\prime}$) and nature of the intervening medium, spacers and/or solvent between redox centers.[7] The literature data indicate that besides the above-mentioned factors local structure and dynamics of protein matrix[2,8-10] and mutual orientation of the centers[4,7,11] also determine the rate and efficiency of the unpaired electron migration. These factors may result in a smaller value of the reaction rate for closely located but unfavourably oriented reagents.

Indeed, it has been observed that the changes in protein conformation induced by denaturation affect the intramolecular electron transfer processes [1,2] strongly. Therefore, the observation of the temperature-dependent rate of intramolecular electron transfer could possibly be used to probe changes in the conformation and conformational dynamics of proteins. In order to check the feasibility of such an approach, hen egg-white lysozyme was selected as a model protein of well-known crystal structure[12] and was used to study extensively: conformation,[13] thermally induced denaturation,[14] and conformational dynamics.[15] It contains a number of potential pulse radiolytically inducible $Trp^{\cdot}$ /Tyr redox-pairs; between some of these

intramolecular electron transfer has been observed.[1,2,4] At least, three of the tryptophans (Trp-62, -63, -108) are located in the active site of the enzyme, and are involved in the binding of oligosaccharide-type substrates.[16] In the predenaturational temperature range up to 60°C, a marked nonlinear temperature dependence of the enzymatic reaction rate had been observed,[17] therefore, one could expect that in the same range of temperatures electron transfers involving at least one of the active site tryptophyl residues would be thermally affected through similar changes in the conformational dynamics of the enzyme. Indeed, it has been demonstrated on the basis of ^{13}C-NMR data[18] that a number of tryptophans and tyrosine residues are involved in thermally-induced structure perturbations occurring in the active site, and in hydrophobic box regions of the lysozyme. This report describes a preliminary pulse radiolysis investigation concerning the effect of temperature on the rate of intramolecular electron transfer between tryptophan and tyrosine residues in lysozyme.

MATERIALS AND METHODS

The lysozyme was from SERVA, and was used as received. All other chemicals (NaN_3, NaH_2PO_4) were of the purest commercially available grade from Merck and were used without further purification. Aqueous solutions of lysozyme (4 mg/ml) containing additionally 10^{-1} M of NaN_3 and phosphate buffer (10^{-3} M) in triply distilled water were prepared immediately before irradiation. The solutions were bubbled with high purity N_2O in order to convert hydrated electrons into hydroxyl radicals. The pH of the irradiated solutions was kept constant at 6.3 in order to initiate tryptophan oxidation by $N_3^{\cdot}$ radicals,[1] in order to observe relatively fast and efficient transformations of tryptophyl into tyrosyl radicals,[1] and finally to prevent lysozyme from becoming denaturable[19] in the range of temperatures (288–333 K) investigated. The pulse radiolysis experiments were performed using a 10 MeV-HRC linear accelerator in Risø National Laboratory by the delivery of single 0.5 μs electron pulses. Radical concentrations were determined by ferrocyanide dosimetry, and doses were in the region of up to 7 Gy per pulse. A thermostated spectrophotometric cell equipped with a water jacket was employed in temperature dependence studies.

RESULTS

Azide radicals ($N_3^{\cdot}$) formed during a pulse react selectively[1,2] with the tryptophyl residues of the lysozyme-generating indolyl-type radicals (reaction 1):

$$N_3^{\cdot} + \text{Lysozyme[6Trp,3Tyr]} \rightarrow N_3^{-} + \text{Lysozyme}$$
$$\text{[xTrp}^{\cdot}\text{,(6-x)Trp,3Tyr]} + H^{+} \qquad (1)$$

The spectra of $Trp^{\cdot}$, $\lambda_{max} = 510$ nm, and of $TyrO^{\cdot}$, $\lambda_{max} = 405$ nm are well separated thus allowing facile observation of the electron transfer process (reaction 2):

$$\text{Lysozyme[xTrp}^{\cdot}\text{,(6-x)Trp,3Tyr]} \xrightarrow{k_2} \text{Lysozyme}$$
$$\text{[yTyrO}^{\cdot}\text{,(x-y)Trp}^{\cdot}\text{,(6-x)Trp,(3-y)Tyr]} \qquad (2)$$

Generally, experimental kinetic traces representing reaction (2) could be satisfactorily described by first-order kinetics.

TABLE I
Intramolecular electron transfer rate constants, yields and percentage of $Trp^{\cdot}$ → $TyrO^{\cdot}$ conversion measured in lysozyme at different temperatures

T (K)	$\bar{k}_2$ s^{-1}	G($Trp^{\cdot}$) initial	G($TyrO^{\cdot}$) initial	G($Trp^{\cdot}$) final	G($TyrO^{\cdot}$) final	Conversion $\Delta G TyrO^{\cdot}/\Delta G Trp^{\cdot}$
288	1.5×10^3					
293	1.6×10^3					
298	1.8×10^3					
303	1.9×10^3					
308	2.5×10^3					
‡		5.70	0.55	0.70	3.7	63%
313	2.8×10^3					
318	3.4×10^3					
323	4.1×10^3					
328	5.3×10^3					
333	8.2×10^3					

$\varepsilon_{510} Trp^{\cdot} = 1.96 \times 10^3$, $\varepsilon_{405} Trp^{\cdot} = 0.4 \times 10^3$, $\varepsilon_{405} TyrO^{\cdot} = 3.2 \times 10^3$, $\varepsilon_{510} TyrO^{\cdot} = 0$ ($dm^3 mol^{-1} cm^{-1}$).[1,2]

‡ The yields were measured for each of the temperatures and were found constant within the experimental error.

The rate constant ($\bar{k}_2$) was determined at a number of temperatures (cf Table 1) from variations in time of the absorbancy at 510 nm and 405 nm. This constant was found to increase more than 5-fold (1.5×10^3 to 8.2×10^3 s^{-1}) within the 288–333 K temperature range.

Assuming in a first approximation equal reactivity of all accessible tryptophan residues with $N_3^{\cdot}$ radicals, and that Poisson's distribution is applicable, it was calculated that under our experimental conditions ([$Trp^{\cdot}$] = 4.0×10^{-6} M, [Lysozyme] = 2.8×10^{-4} M) more than 99% of the oxidized lysozyme molecules contain only a single tryptophyl radical. Moreover, $\bar{k}_2$ was found to be invariant towards a five-fold increase in the lysozyme concentration. Therefore, the measured rate constant represents an effective rate constant of intramolecular electron transfer occurring between only one of the six tryptophans and any of three tyrosyl residues in the molecule. Since the kinetic data conform satisfactorily to the single exponential process, the observed electron transfer involves either one $Trp^{\cdot}$/Tyr pair or more, characterized by nearly the same rate constants.

An inspection of calculated G-values (cf Table 1) indicates that G ~ 5.7 for oxidation of tryptophans is apparently constant over the whole range of temperatures investigated. This is due to a complete scavenging of $N_3^{\cdot}$ radicals by lysozyme followed by quantitative tryptophan oxidation. Under these circumstances the constant 63% efficiency of tryptophyl-tyrosyl radical transformation indicates the presence of other, parallel to eq. (2) route for the disappearance of $Trp^{\cdot}$ characterized by an activation energy close to that of reaction (2). If this is the case the real rate constant for reaction (2) equals 63% of the experimental $\bar{k}_2$ value.

A standard Arrhenius plot of $\ln \bar{k}_2$ vs. T^{-1} obtained according to (3) is shown in Figure 1:

$$\ln \bar{k}_2 = 1 + \ln (^{KT}/h) + \frac{\Delta S^{\#}}{R} - \frac{E_a}{RT} \qquad (3)$$

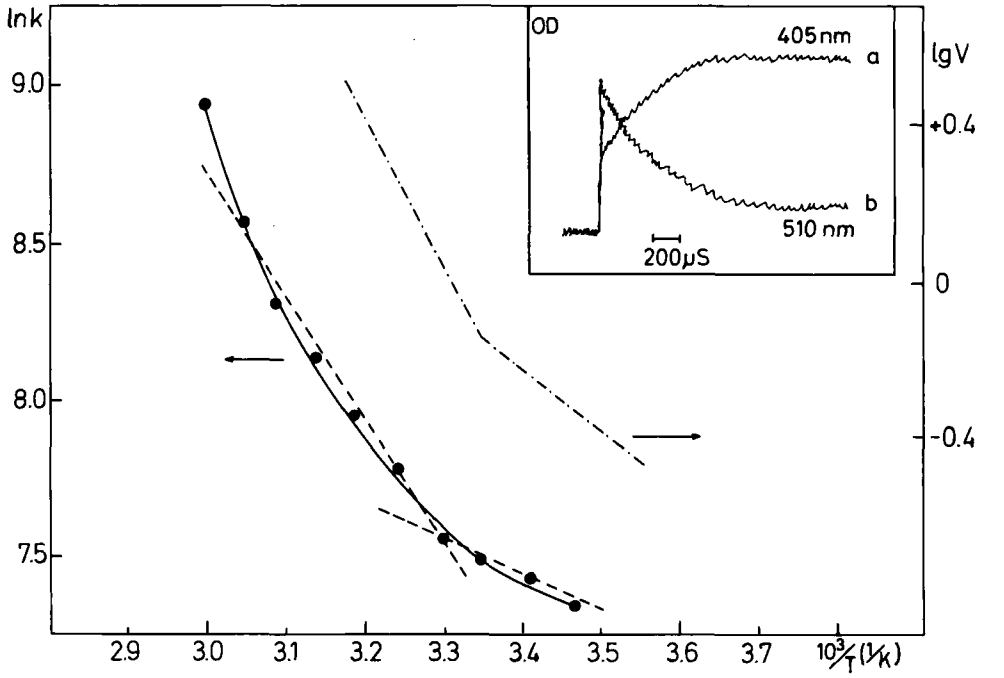

FIGURE 1 (- - -●- - -) Arrhenius plot for the temperature dependence of the intramolecular electron transfer involving tryptophan and tyrosine residues in hen egg-white lysozyme molecule. (-·-·-) Arrhenius plot established from kinetic determinations of bacterial substrate digestion by lysozyme.[16] Inset: Absorption-time curves obtained at 405 nm a) and at 510 nm b) in pulse radiolysis of 2.8×10^{-4} M lysozyme in the presence of 10^{-1} M NaN_3 and 10^{-3} M phosphate buffer at 25°C.

The plot exhibits a sharp break at about 303 K. It can be approximated by two straight lines of different slopes which correspond to activation energies: $E_a = 12.5\,kJ\,mol^{-1}$ and $E_a = 31.0\,kJ\,mol^{-1}$ in the ranges 288–303 K and 303–333 K, respectively. The entropy of activation calculated at 298 K for a process characterized by lower E_a is large and negative ($\Delta S^{\#}_{298} = -150\,J\,deg^{-1}\,mol^{-1}$). This confirms the necessity that the mutual orientation of the reacting groups in the electron transfer observed[4] shall be set properly.

DISCUSSION

In the light of the current theory of electron transfer processes in proteins,[7] the increase in the rate of electron transfer between fixed members of some Trp/Tyr pairs in lysozyme observed upon a rise of temperature and associated with a large and negative entropy of activation, results for the most part from thermally induced motions in the protein matrix. These motions allow for a larger range of mutual orientations of tryptophan and tyrosine residues including those at which an efficient electron transfer may occur.

The most characteristic finding, however, is a close similarity between Arrhenius

plots (Figure 1) for the kinetics of electron transfer in lysozyme and for the kinetics of its enzymatic reaction[17] characterized by a sharp break at about 298–303 K. This indicates that the same thermally induced conformational changes in lysozyme are responsible for the observed temperature dependence of the two processes. Moreover, these changes must affect conformational motions of tryptophan and/or tyrosine residues engaged in the binding of substrates or located in the proximity of substrate binding sites in the active center of the enzyme.

The sharp break at the start of the 298–303 K range in the Arrhenius plot for the enzymatic reaction,[17] a phase transition involving the transforming of the tetragonal to orthorhombic crystal form of lysozyme at temperatures of about 298 K,[20] as well as a number of other temperature-dependent properties of lysozyme in solution in the predenaturational range of temperatures (cf[18] and references cited therein) all suggest the existence of two distinctly different conformations in which lysozyme behaves differently. This interpretation does not find any support in calorimetric data for thermal behaviour of lysozyme,[21] however. In the predenaturational range of temperatures no heat absorption was observed,[21] so that the occurrence of a temperature-induced cooperative conformational transition must be excluded. We are thus tempted to interpret nonlinearity of the Arrhenius plots for the kinetics of both processes as a result of predominance at low temperatures of low-energy barrier motions within the substrate binding domains of the lysozyme molecule. With a further rise of temperature the low-energy barrier motions increase in amplitude, allowing thus motions hindered by higher energy barriers. Indeed, crystallographic structural data,[13] ^{13}C NMR conformational investigations,[18] and molecular dynamic studies of internal motions in hen egg-white lysozyme[15,22] lend strong support to this interpretation. They show that within the substrate binding domains of lysozyme, a number of tryptophan and tyrosine residues differ substantially in the amplitude of their thermal motions in the normal range of temperatures. According to the hinge-bending model of thermal motion in the binding cleft of lysozyme,[23] they involve two globular lobes between which the cleft containing the active enzyme site is located. In the first lobe the following potential redox-pair members appear: Tyr-20, Tyr-23, Trp-28, Trp-108, Trp-111, and Trp-123, while in the other: Tyr-53, Trp-62, and Trp-63. Of these Trp-62 and Trp-111 exhibit particularly high root mean square fluctuations as estimated both from crystallographic temperature factors,[12] and molecular dynamics simulation.[15] The motions of the remaining tryptophans are more restricted due to the high number of close contacts formed with back-bone and side-chains atoms located in their environment.[15] In the light of presented structural and dynamic data, the following tryptophan/tyrosine pairs, which potentially could contribute to the observed electron transfer in the lower temperature range can be proposed: Trp-62/Tyr-20, Trp-62/Tyr-23, and/or Trp-111/Tyr-53, as composed of residues located in the opposite lobes.

According to the hinge-bending model considered in the higher temperature range, the following more sterically hindered tryptophan/tyrosine pairs could possibly contribute to electron transfer processes: Trp-63/Tyr-20, Trp-63/Tyr-23, and/or Tyr-53 and either one of the tryptophans: Trp-28, Trp-108, and Trp-123.

In order to decide for which of the above proposed Trp/Tyr pairs the electron transfer could really occur, the distances between the involved residues are to be considered in relation to rate constant-distance dependence. The latter was determined from a series of Trp-$(Pro)_n$-Tyr peptides.[4] The rate constants comparable to those observed in lysozyme were found for Trp-Pro-Pro-Pro-Tyr peptide. Assuming

TABLE II
Distances between all possible Trp/Tyr pairs in lysozyme derived from crystallographic data[12]

Tryptophan	Tyrosines	Distances[a]
Trp-28	Tyr-20	9.46 A
	Tyr-23	8.65 A
	Tyr-53	23.71 A
Trp-62	Tyr-20	18.87 A
	Tyr-23	17.67 A
	Tyr-53	13.24 A
Trp-63	Tyr-20	13.89 A
	Tyr-23	14.45 A
	Tyr-53	12.78 A
Trp-108	Tyr-20	13.59 A
	Tyr-23	11.38 A
	Tyr-53	14.68 A
Trp-111	Tyr-20	16.02 A
	Tyr-23	7.23 A
	Tyr-53	24.10 A
Trp-123	Tyr-20	22.03 A
	Tyr-23	15.51 A
	Tyr-53	26.70 A

[a]all distances measured as center to center imidazole N-phenolic O-atom.

that the separation distance is 12.5 A between Trp and Tyr residues (center-to-center imidazole's N-phenolic O) for fully extended all-trans conformation of the Trp-Pro-Pro-Pro-Tyr peptide in aqueous solutions, we can set the limiting distance for the observable electron transfer in lysozyme at this same value. Taking this limiting distance into account, inspection of the individual distances between all possible Trp/Tyr pairs derived from crystallographic date[12] (Table 2) allows us to select only four effective redox pairs: Trp-28/Tyr-20, Trp-28/Tyr-23, Trp-108/Tyr-23, and Trp-111/Tyr-23. These pairs are all localized in one of the substrate binding lobes of lysozyme. Therefore, the intra- rather than interlobe fluctuations can be held responsible for the temperature dependence of the electron transfer rate. The presence among these pairs of both types of tryptophan, e.g. highly mobile Trp-111 and with strongly hindered motions Trp-28, Trp-108 is in accordance with our interpretation of the Arrhenius plot.

The more detailed interpretation will require further experimental support. In this connection investigations concerning the effect of substrate binding to lysozyme, chemical or site-mutagenic removal of particular tryptophans and/or tyrosines, as well as oxidation of tryptophans by radicals of different selectivity may prove to be of great advantage.

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