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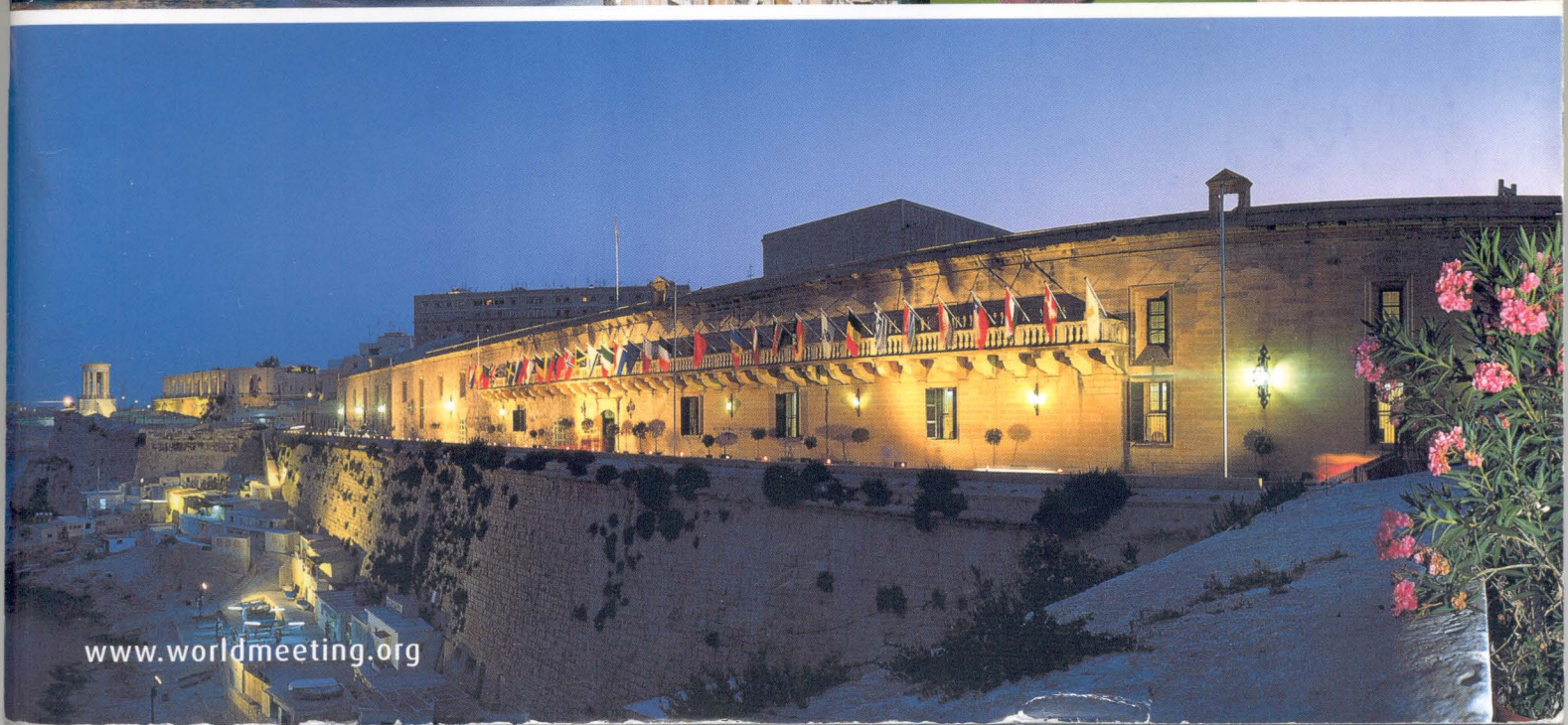

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EFFECT OF POLOXAMER 407 ON THE STABILITY AND ENZYMATIC ACTIVITY OF YEAST ALCOHOL DEHYDROGENASE.

Stefania Pucciarelli[1], Giulia Bonacucina[2], Franco Bernabucci[1], Marco Cespi[2], Giovanna Mencarelli[2], Giusi Serena De Fronzo[1], Giovanni Filippo Palmieri[2]

[1]Department of Comparative Biochemical and Morphological Sciences, Via Gentile III da Varano, University of Camerino
[1]Department of Chemical Sciences, Via S. Agostino 1, University of Camerino

INTRODUCTION

The delivery of proteins and peptides is gaining an increasing interest in the last few years but the development of a suitable drug delivery system is still an important issue since many obstacles can be encountered in the administration of proteins and peptides in particular for oral formulation. Proteins are large molecules with complex structures characterized by labile bonds and side chains with reactive groups. Possible disruption of these bonds or modifications of the side chains can bring to loss of activity.
Ideal drug delivery systems are controlled release formulations, that can avoid the problems of large doses or multiple injections with consequent unwanted side effects and the necessary presence of medical supervision.
Block copolymers of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) represents a class of thermoresponsive polymers materials. The presence of PEO and PPO blocks in a single polymer chain gives rise to essentially amphiphilic molecules whose self-assembling properties display a wide range of phase behaviour. This ability to form micelles and liquid crystalline phases is strongly temperature dependent (1). Despite a large number of studies are present in the literature concerning the protein release, there is a significant lack on the knowledge about possible modifications of the proteins structure inside the delivery systems. The weak structural equilibrium of a protein molecule can be influenced by the loading into a gel structure or after the release and the native state can be converted in a less stable intermediate state or in an unfolded state. The aim of this study is to analyze the interaction between Poloxamer 407 and yeast alcohol dehydrogenase (ADH), chosen as model protein. Alcohol dehydrogenases in general are temperature labile, and thermoinactivation of yeast ADH primarily involves intersubunit interactions before thermal unfolding (2). In the present investigation enzymatic activity assays and differential scanning calorimetry analysis were carried out to characterize the interaction between Poloxamer 407 and yeast alcohol dehydrogenase. By performing a pH screening we could relate the effect of Poloxamer 407 to the conformational changes induced by acidic and alkaline pH values.

EXPERIMENTAL METHODS

Materials

POLOXAMER 407 (LUTROL®F-127) was from BASF Chem.Trade Gmbh (91593 Burgbernheim - Germany).
Alcohol dehydrogenase (ADH) from baker's yeast, ?-NAD, TRIS, Glycine Potassium Phosphate, Potassium Acetate were from Sigma Aldrich Co. Ltd. (Gillingham, Dorset SP8 4XT U.K.)

Samples preparation

Poloxamer solutions were prepared by dispersion of the solid material in the required amount of buffer using the "cold" procedure. The Poloxamer samples containing ADH were prepared by adding the enzyme in the buffered Poloxamer solutions. ADH concentration was 0.1 mg/ml in the kinetic assays and 10 mg/ml in the calorimetric studies. Samples were then stored at 4 °C for at least 24 hours before analysis.

Differential scanning calorimetry (DSC)

The calorimetric studies were carried out with a micro DSC III Setaram in 0.85 ml cells. Samples were loaded in the calorimetric cells at the temperature of 0° and heated at 0.6 °C/min to 110 °C. All samples were run in triplicates, by subtracting the baseline either run with buffer against buffer, or Poloxamer against Poloxamer. The yeast ADH concentration in all the scans was 10 mg/ml and the quantity of sample loaded was 750 mg. The transition temperature T_m was determined from the peak apex temperature and the transition enthalpy, ?H, when possible, was measured by integrating the peak area in the thermogram.

Enzyme stability

The effects of Poloxamer 407 on ADH catalytic stability were assayed by incubation of copolymer with the enzyme for fixed time intervals at 37 °C, removal of small aliquots of the incubation mixture and assay of enzyme activity under saturating substrates concentrations. The enzyme activity assays were designed to dilute the Poloxamer (1:20)

in order to avoid the potential effects of copolymer on enzyme catalytic activity during the assay.

Steady-state kinetic parameters measurement

Enzymatic activity was determined in a double-beam spectrophotometer (UVIKON 940, Kontron Instruments) at 30 °C and pH 9.1, using the method reported by Bergmeyer H. U., that measures the absorbance increase at 340 nm due to reduction of NAD to NADH. In all our experiments the enzymatic Unit is defined as the amount of enzyme required to convert 1 ?mol of ethanol into acetic aldheyde in one minute at pH 9.1 and 30 °C.

The yeast ADH is a tetrameric enzyme with four independent active sites. In this work the steady-state kinetic analysis has been approached by using the Michaelis-Menten theory. The effects of Poloxamer 407 on enzyme catalytic activity were determined.

RESULTS AND DISCUSSION

Calorimetric analysis

The results obtained by DSC scans of yeast ADH in absence and in presence of 2% Poloxamer 407, at different pH values, are summarized in Table I. At pH 9.1 a scan also in presence of 20% Poloxamer was performed.

Table I: Effect of Poloxamer 407 on the thermal unfolding parameters measured by DSC

	0%	0% (o/n at 4 °C)	2%	20%
pH 9.1				
T_m	46.6	46.3	42.5	48.7
?H	183.4	87.6	215	nc
pH 7.3				
T_m	61.7	62.2	62.5	
?H	220.2	195	471	
pH 4.78				
T_{m1}	54.6	52.6	np	
$?H_1$	35	39	np	
T_{m2}	61	61.2	62	
$?H_2$	78.8	107.1	nc	

T_m is expressed in °C and ?H in kcal/mol
nc = non calculated, np = non present

The main effect exerted by Poloxamer 407 on the thermal denaturation of ADH is represented by the recovery of the transition enthalpy both at pH 9.1 and 7.3, probably through the stabilization of intersubunit interactions.

Enzyme stability

The effect of Poloxamer 407 on ADH enzymatic thermostability was pH dependent: at pH 9.1 the copolymer had a protective effect against inactivation at both 10% and 20% concentrations; at pH 7.3 the protective effect was at a lower extent than at pH 9.1; in contrast, at pH 4.78 the presence of Poloxamer made the enzyme more sensitive to the thermoinactivation process.

We have measured the enzymatic activity of ADH freshly dissolved and then the effect of Poloxamer on the stability has been monitored after overnight at 4 °C to allow the Poloxamer 407 to stabilize the interaction with the protein.

In absence of Poloxamer the pH 9.1 had a strong effect on enzyme activity after o/n at 4 °C: ADH activity was reduced from 206.6 U/ml to 14.14 U/ml in absence of Poloxamer, to 43.26 U/ml in presence of 10% and 175.83 U/ml in presence of 20% Poloxamer. Then we observed the behaviour at 37 °C, shown in Figure 1:

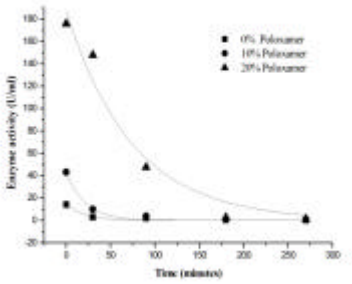

Figure 1: Enzymatic stability of ADH at 37 °C.

In presence of 20% Poloxamer the thermoinactivation half time $t_{1/2}$ increased from 21 to 74 minutes, at pH 9.1.

Steady-state kinetic parameters determination

By using the Michaelis-Menten equation we have obtained the kinetic parameters for the reaction catalyzed by yeast ADH at pH 9.1 and 30 °C. The effect of Poloxamer 407 in samples kept over/night at 4 °C is reported in Table II.

Table II: Effect of Poloxamer 407 on steady-state kinetic parameters

% Poloxamer	$K_{m(EtOH)}$ (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m (M^{-1} sec^{-1})
0	12.93	150	$1.16 \cdot 10^4$
2	10.41	151	$1.45 \cdot 10^4$
5	8.636	200	$2.32 \cdot 10^4$

The kinetic parameters of the reaction catalyzed by ADH were not affected by the copolymer when determined one hour after samples preparation (data not shown). Thus, the effect of Poloxamer 407 on the catalytic behaviour of ADH is exerted at structural level, by helping the enzyme in maintaining the proper active site conformation, rather than by altering the catalytic mechanism.

References

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