Biochimica et Biophysica Acta, 364 (1974) 146–158

BBA 67290

ENZYMATIC ACTIVITY AT INTERFACES

I. ENZYMATIC ACTIVITY OF TRYPSIN AT THE AIR–WATER INTERFACE

L. INBAR* and I. R. MILLER
Polymer Department, Weizmann Institute of Science, Rehovot (Israel)
(Received April 1st, 1974)

SUMMARY

In order to study the enzymatic activity of adsorbed and spread monolayers of trypsin, its adsorption isotherm and spreading tendency have been determined using radioactively labelled trypsin. The labelling of trypsin by acetylation did not affect its biological activity and neither did it appreciably alter its surface activity.

The enzymatic activity of trypsin spread on the adsorbed surface layers was considerably lower than its activity in the bulk phase. The activity decreased with decreasing surface concentration and with added spreading agent, e.g. isopropanol. Displacement of the enzyme from the surface by the highly surface-active substrate did not recover its biological activity.

These experiments, together with the measured dependence of surface tension on protein concentration, indicate very strongly that the major part of the trypsin in the surface layer is irreversibly adsorbed and that its tertiary structure is substantially altered.

INTRODUCTION

A large portion of biological activity involving different enzymes takes place at the interface between membranes and aqueous solutions. There are, however, other interfaces like air–water or hydrocarbon–water interfaces which tend to inactivate some enzymes. Unfortunately, investigation of the structure and function of proteins is much more difficult at interfaces than in the bulk of the solution, especially when a simultaneous investigation of both is attempted. Optical measurements, e.g. optical rotatory dispersion or circular dichroism, are most suitable for conformational analysis in solution. However, such measurements cannot be carried out on monolayers at all. Other optical measurements like light absorption or fluorescence have a very low accuracy when applied to monolayers at liquid interfaces and are of little practical use. The infrared attenuated total reflection spectra of protein and polypeptide monolayers transferred on germanium prisms indicate [1] that the secondary structure,

* Part of the Ph.D. Thesis, submitted to the Feinberg Graduate School.
Abbreviation: Bz-Arg-ONAn, N^{a}-benzoyl-DL-arginine *p*-nitroanilide hydrochloride.

namely, the degree of helicity, is only slightly affected by adsorption or spreading at the water–air interface. If the surface forces do affect the biological activity of the enzymes, they are bound to do so by changing their tertiary structure. We decided, therefore, to investigate the activity of enzymes differing in their structural stability when spread at the air–water interface. The first paper of this series deals with the enzymatic activity of trypsin in surface layers. When studying the activity of enzymes in monolayers, any activity of enzymes dissolved in the subphase has to be either completely eliminated or at least satisfactorily accounted for. It has been shown in our experiments as well as by other investigators, studying enzymatic activity in monolayers [2, 3], that it is impossible to eliminate dissolution of the enzyme during spreading. We, therefore, resorted to successive exchange of the solution in the subphase, before adding the substrate, until the total amount of enzyme in the subphase was negligible with respect to the enzyme content of the monolayer. To ensure this we had to be able to determine quantitatively minute amounts of enzyme both in the subphase and on the surface. This has been achieved by using tritium-labelled enzymes. Even if the enzyme was not completely eliminated from the subphase its activity could be determined in a control experiment and corrected for. Moreover, by the determination of minute concentrations in the subphase, the specific activity of the enzyme dissolved from the monolayer into the subphase could be determined.

EXPERIMENTAL

Materials

Bovine pancreas trypsin twice recrystallized and lyophilized, was purchased from Worthington Biochemical Corp. We also purchased trypsin derived from the same source which had been tritiated by exposure to tritium (Wilzbach method) from the same company. Its radioactivity was 40 μCi/mg, which was adequate, but its enzymatic activity was only 8% of the activity of the non-radioactive substance from which it was prepared. The low enzymatic activity made the labelled product unsuitable for the investigation of surface forces on the enzymatic activity. Moreover, a 92% reduction of enzymatic activity through tritiation by autionization as in the Wilzbach method casts doubt as to whether the radioactive and the biologically active molecules are identical.

Radioactive trypsin by acetylation

Trypsin labelled by this method [4] retains its complete enzymatic activity. The details of the method are described elsewhere [4]. The method is based on the acetylation by tritium or ^{14}C-labelled acetic anhydride at 0 °C after the lyophilized protein has been wetted by the anhydride in benzene solution. 0.7 ± 0.05 acetyl groups per protein molecule on the average was incorporated into the trypsin used here. Acrylamide gel electrophoresis indicated that the distribution of the acetyl label was very uniform and only a negligible number of molecules bound more than one acetyl group. However, to be absolutely certain that the measured radioactivities on the surface and in the bulk truly represent the concentrations in the two phases, we had to prove that there is no enrichment of the label in one of the phases. This has been done by mixing the labelled with non-labelled trypsin and determining the adsorption isotherms of the different mixtures (see Results).

The substrate N^{α}-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (Bz-Arg-ONAn) was purchased from Schwarz and Mann. The tritium-labelled cholesterol and oleic acid used for the calibration of the surface counting was purchased from Radiocentre, Amersham, Buckinghamshire.

Methods

Spreading of the enzyme

The enzyme was spread at the air–water interface in a trough made of stainless steel and subdivided into four compartments (Fig. 1). The partitions between the

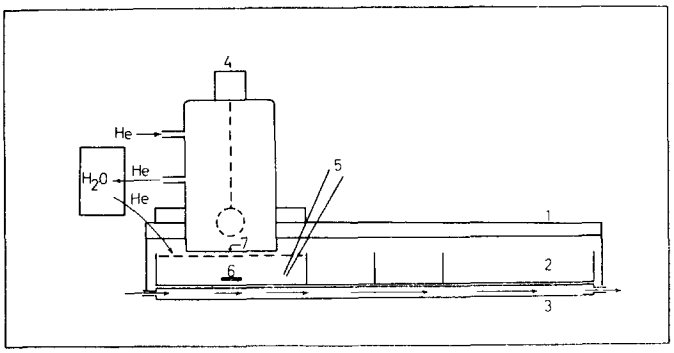

Fig. 1. Trough and Counter. 1, cover; 2, four-compartment trough; 3, thermostating; 4, gas-flow counter with ultrathin end window; 5, tube for adding enzyme or substrate and for exchanging the subphase underneath the monolayer; 6, magnetic stirrer.

compartment had a sharp edge on the top. The two end compartments (I and IV) were 5 cm × 5 cm, while the two middle compartments were 5 cm × 2.5 cm. In the preliminary experiments, the trough was filled until about 0.5 mm below the edges which were wetted by a thin film of the aqueous solution. The enzyme was spread over a glass sphere (Trunit method) in Compartment II. Since the contact between the compartments was maintained only through a thin surface layer, it was hoped that no dissolution from the enzyme surface layer will occur in Compartment I after its "washing" in Compartment II. Just as in the older experiments by Skou [2], dissolution occurred in all the parts of the trough.

After it transpired that the dissolution from the surface layer during spreading is unavoidable and that the dissolution from the surface diminishes with the age of the spread layer, we decided to spread the enzyme on the surface of Compartment I. To facilitate spreading and to reduce dissolution of the enzyme, 20% isopropanol was added to the aqueous spreading solution. Nevertheless, about 80% of the protein applied was dissolved in the subphase. Most of the protein was removed from the subphase by the consecutive exchange of the subphase by new buffer solution. This was done by subsequent removal of half the subphase through a tube permanently installed in the through compartment and its replacement by the same amount of buffer solution. This process, accompanied by stirring of the biphase, was repeated until the total amount of enzyme in the subphase was less than 5% of its amount in the surface layer. At this point half the subphase was transferred into Compartment

IV to serve as a blank for the enzymatic activity in the subphase. The substrate was then injected into the subphase and into the blank compartment. The enzymatic activity was determined by measuring the light adsorption at $\lambda = 400$ nm.

The surface concentration of the tritium-labelled trypsin was measured with a gas flow counter equipped with a 2000–5000-Å thick formvar end window [5]. The counter was calibrated by spread monolayers of tritium-labelled cholesterol or oleic acid. To eliminate condensation of water vapour on the end window, a temperature gradient was maintained by cooling the trough. The concentration of the labelled protein in the subphase was determined by scintillation counting of the aliquots.

The surface pressure of the protein layers was determined by an automatic Wilhelmy type surface balance built in our laboratory [6].

RESULTS

Calibration of the end-window counter

The efficiency of the tritium counting from the surface depends on the thickness of the end window, which varied between 2000–5000 Å, and on the density of the gas within the gap between the water surface and the counter and on the thickness of this gap. In our device this gap is flushed with Q gas, which is about 99% He. The procedure increased the efficiency of the counting, diminished the sensitivity to the

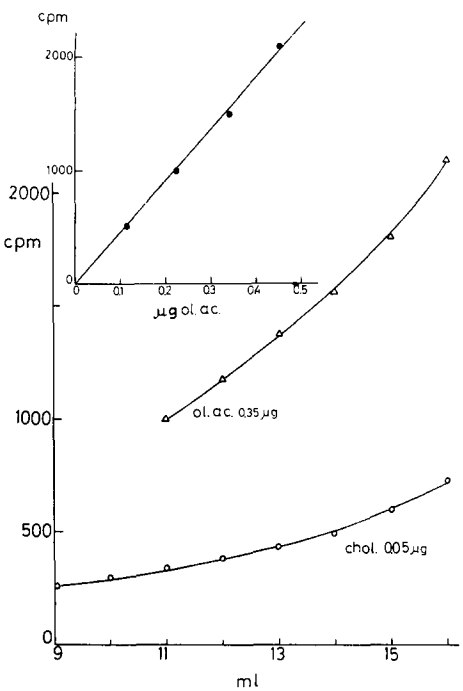

Fig. 2. Surface count from monolayers of oleic acid (ol.ac.) and cholesterol (chol.) as a function of the distance between the counter and the surface expressed as the volume of subphase in Compartment I underneath the counter. Insert: Surface count as a function of the surface concentration of oleic acid expressed in μg per area of Compartment I (25 cm^2). The volume of the subphase in the compartment was 14 ml.

distance between the counter and the surface and, last but not least, reduced the chances for undesirable gas molecules to diffuse through the very thin window into the counter. However, even under these conditions the counting efficiency depended on the distance between the window and the surface. In the course of these experiments, which lasted for many hours, this distance changed due to evaporation from the subphase which was facilitated by the flushing Q gas and could not be completely eliminated even by partial presaturation of the gas with water vapour and by cooling the trough a few degrees below room temperature (Fig. 1). The initial distance between the surface and the counter window was set by the volume of aqueous solution in Compartment I. The area of this compartment was 25 cm^2 so that changing the volume by 1 ml changed the distance between the surface and the counter by 0.4 mm. In Fig. 2 the calibrating curve which shows the dependence of the counting efficiency on the distance is presented. From the calibration curves the thickness of the He atmosphere required to reduce the surface radiation to half was determined to be 1.9 ± 0.1 mm. Assuming this gas to be saturated with water vapour at 24 °C, its density should be 0.22 mg/ml. The adsorbing mass of a 1.9-mm thick layer was $\varrho_s = 42\ \mu g/cm^2$. The mass adsorption coefficient of the including impurities like water vapour and air is about $\mu = 12\ cm^2/mg$ [7]. The attentuation factor η_a is expressed approximately by

$$\eta_a = \exp(-\mu\varrho_s)$$

For η_a to be 0.5, $\mu\varrho_s$ is 0.69 or ϱ_s is approx. $57 \pm 2\ \mu g/cm^2$. This indicates that the Q gas within the gap between the surface and the counter was not only saturated with water vapour but also about 5% of its volume was air.

For a fixed distance between the counter and the surface the count is proportional to the surface concentration (Fig. 2, insert). The counting efficiency from the surface was obtained by comparison with the scintillation counting of the same amount of spread oleic acid. The surface concentration Γ_p of the spread or adsorbed enzyme is then given by the relation

$$\Gamma_p = \left(\frac{\text{Scintillation count}}{\text{Surface count oleic acid}}\right)\left(\frac{\text{Surface count}}{\text{Scintillation count protein}}\right)\left(\frac{\text{Wt of protein for Scintillation count}}{\text{Surface area}}\right)$$

Adsorbed and spread surface layers of trypsin

The adsorption of different mixtures of labelled and non-labelled trypsin from the aqueous solution onto the air–water interface was measured. The main purpose was to see to what extent the acetylation affects the surface activity of the protein. As is evident from Fig. 3, the surface count is proportional to the fraction of the labelled part in the protein mixture, which indicates that the proportion of the labelled to the non-labelled protein in the bulk and in the surface is the same and there is no appreciable change in surface activity by acetylation. This behaviour of the acetylated trypsin seems to differ from that of acetylated lysozyme [8] where the surface pressure has been shown to increase with acetylation. However, no data has been presented on the competitive adsorption of the acetylated and of the intact lysozyme.

In Fig. 4 the adsorption isotherm of trypsin at pH 7.9 is given. The surface

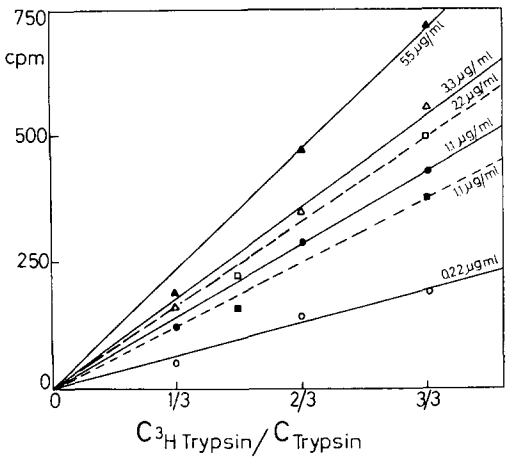

Fig. 3. Surface count as a function of the ratio of labelled (by acetylation) to unlabelled trypsin. The total concentrations of trypsin in the subphase are indicated in the figure. Full lines, pH 7.9; broken lines, pH 3.

concentration increases very steeply up to a bulk concentration of about 1 μg/ml. A surface saturation plateau of about 0.12 μg/cm^2 is approached at a bulk concentration of 3 μg/ml. This surface concentration corresponds to an area of 3300 Å^2 per molecule of trypsin, or about 15 Å^2 per amino acid residue. Another flat adsorption step can be observed around a bulk concentration of 5 μg/ml but no surface saturation is reached up to 10 μg/ml. At this point the surface concentration reaches the value of about 0.18 μg/cm^2 and the average area per trypsin molecule becomes 2200 Å^2, which makes about 10 Å^2 per amino acid residue. These surface concentrations correspond to more than a monolayer. Evidently after the completion of a monolayer at a very low bulk concentration, further adsorption is controlled by a considerably lower standard free energy of adsorption.

The surface tension lowering of an aqueous solution of 0.05 M phosphate at pH 8 has also been measured. As seen from Fig. 5, the surface tension varies appre-

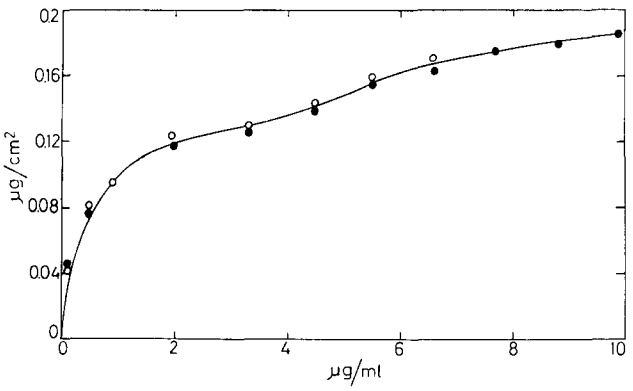

Fig. 4. Adsorption isotherm of trypsin at pH 7.9 in 0.04 M phosphate buffer. Open circle, trypsin labelled by acetylation only. Filled circle, labelled trypsin diluted with the original unlabelled trypsin at a ratio of 2:1.

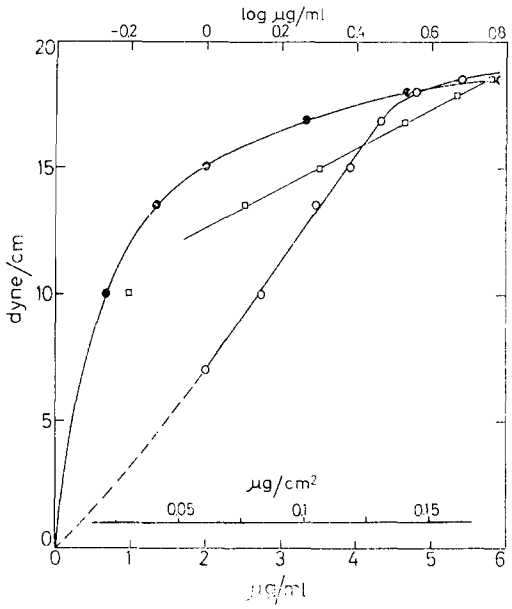

Fig. 5. Surface tension lowering as a function of bulk concentration (filled circles), of surface concentration (open circles), of trypsin 0.04 M phosphate buffer (pH 7.9), and of log bulk concentration (open squares).

ciably with the concentration of trypsin in the whole concentration region. At low concentrations there is some doubt whether the equilibrium values of the surface tension have been reached. Thermodynamically more significant values are expected in the region where the direct measurements of surface concentration indicate that a limiting surface concentration has been reached. In this region the surface tension varies linearly with the logarithm of the protein concentration. The area per molecule of trypsin calculated from this slope according to the ordinary Gibbs adsorption isotherm is about 130 Å^2. It is clear that this figure is in complete disagreement with the values of 3300 Å^2 at 3 μg/ml or 2600 Å^2 at 6 μg/ml obtained by the direct measurement of surface concentration. The Gibbs adsorption isotherm can be applied, however, only if the surface layer as a whole is in equilibrium with the bulk phase. This condition does not hold generally in the case of proteins. As has been pointed out previously [8], that part of the monolayer may apparently be irreversibly adsorbed. The interchange of the irreversibly adsorbed part of the monolayer with the part adsorbed reversibly is slow compared with the rate of the reversible adsorption from the bulk of the solution. The Gibbs adsorption isotherm becomes in this case analogous to that of an adsorbed monolayer penetrating a spread monolayer [9] and the surface pressure $d\pi$ is given

$$d\pi = \Gamma_{ir} d\mu_{ir} + \Gamma_{rev} d\mu_{rev} \quad (1)$$

where the subscript ir and rev characterize the surface concentrations Γ and the chemical potentials μ of irreversibly and reversibly adsorbed components.

$$d\mu_{ir} = RT d\ln \Gamma_{ir} + A_{ir} d\pi \quad (2)$$

A_{ir} is the area occupied by an irreversibly adsorbed protein molecule and $\Gamma_{ir}A_{ir}$ is the area fraction of these components in the surface.

For a spread monolayer ($\Gamma_{rev} = 0$) Eqns 1 and 2 give after integration

$$\pi = -\frac{RT}{A_{ir}} \ln (1 - A_{ir}\,\Gamma_{ir}) \tag{3}$$

when Γ_{ir} is constant

$$\frac{d\pi}{d \ln c_p} = \frac{RT\,\Gamma_{rev}}{(1 - \Gamma_{ir}\,A_{ir})} \tag{4}$$

The linear dependence of π on $\log c_p$ in Fig. 5 would indicate, according to Eqn 4, that Γ_{rev} also reached a constant value. However, this is in contradiction to the directly measured increase of the total surface concentration $\Gamma_{ir} + \Gamma_{rev}$.

Assuming that the product $\Gamma_{ir}A_{ir}$ is constant, which implies that the surface compression is matched by the change in configuration, we obtain from Eqns 1 and 2.

$$\frac{d\pi}{d \ln c_p} = \frac{RT}{1 - \Gamma_{ir}\,A_{ir}} \left(\frac{d\Gamma_{ir}}{d \ln c_p} + \Gamma_{rev}\right) \tag{5}$$

where $(d\pi/d\ln c_p)$ from Fig. 5 is 3.2 dyne and $d\Gamma_{tot}/d\ln c_p$ in this region, as obtained from Fig. 4, is about $1.6 \cdot 10^{12}$ molecule/cm^2. Assuming that $d\Gamma_{tot}/d\ln c_p = d\Gamma_{ir}/d\ln c_p$ and that Γ_{rev} is negligible, we estimate, according to Eqn 5, that the fraction of the surface available for equilibrium adsorption $(1 - \Gamma_{ir}A_{ir})$ is $2 \cdot 10^{-2}$. Of course, Γ_{rev} may not be negligible and $(d\Gamma_{ir}/d\ln c_p)$ may be only part of the total change in surface concentration. If Γ_{rev} is a large fraction (e.g. > 0.7) of the surface concentration, $(1 - \Gamma_{ir}A_{ir})$ approaches its maximal value of about $5 \cdot 10^{-2}$. In any event $(d\pi/\ln c_p)$ does not need to be constant over the whole range. Thus the estimate of $(1 - \Gamma_{ir}A_{ir})$ is only qualitative giving the lowest possible value.

Spreading trypsin from aqueous solutions by the Trunit method (Fig. 6, Curve a) results in limiting surface concentrations similar to those obtained by adsorption till the first plateau. Any added quantity to the surface is washed out during the flushing of the subphase and could have been only reversibly adsorbed. When 20% isopropanol was added to the spreading solution, 4–5 times higher limiting concentrations were obtained (Fig. 6, Curve b). At the same time variation in the salt concentration in the subphase had only very little effect on the spreading of the surface layer. With isopropanol as a spreading agent, surface concentrations of above 0.5 μg/cm^2 can be achieved, which corresponds to 800 Å^2 per trypsin molecule. The surface concentrations given in Fig. 6 were obtained after consecutive washing of the subphase until the bulk concentration was below 0.002 μg/ml per 0.1 μg/cm^2 in surface concentration (e.g. when the surface concentration was 0.5 μg/cm^2 the bulk concentration was below 0.01 μg/cm^3).

It is very likely that isopropanol does not just facilitate spreading but causes the surface force to alter the conformation of the protein molecules. In bulk, 20% of isopropanol does not affect the enzymatica ctivity of the trypsin. It can, therefore, be assumed that its effect alone on the tertiary structure of the active site of trypsin is

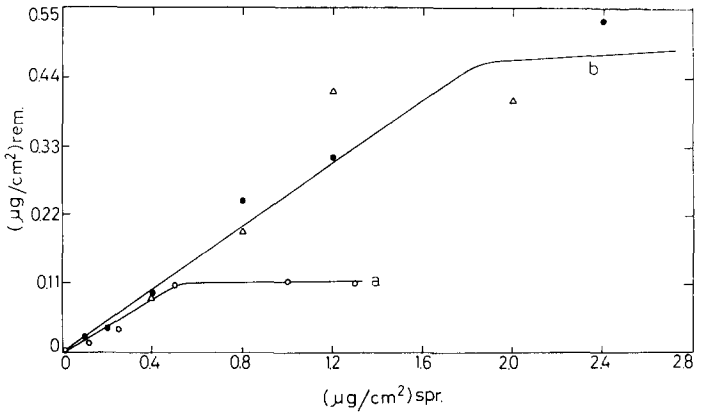

Fig. 6. The dependence of the final surface concentration after washing the subphase on the amount spread per cm². (a) without spreading agent. (b) with 20% isopropanol in the spreading solution.

not very pronounced when in solution. There were two questions that we wished to ask: (1) How does the surface affect the enzymic activity of trypsin? (2) Does the presence of isopropanol during spreading influence the surface effect?

Enzymic activity of adsorbed monolayers

In all the measurements of enzymatic activity, we worked with a large constant excess of substrate (Bz-Arg-ONAn) 0.1 mg/ml with respect to enzyme in order to obtain the maximal possible rates from the surface layers containing small quantities of weakly active enzymes. Thus we obtained only the turnover per enzyme molecule but not the Michaelis constant.

The enzymatic activity of the adsorbed monolayers was measured as follows. Two gas-flow counters were located over Compartments I and IV of the trough (Fig. 1). 15 ml of solution of trypsin at pH 7.9 at low concentrations (between 0.4 μg/ml to 2 μg/ml) was inserted in Compartment I and adsorption was measured for about one hour until it did not vary with time. At this point, half (7.5 ml) of the subphase was removed into a glass bottle and 7.5 ml of new enzyme solution was inserted into Compartment IV to equal volumes of substrate solution added beforehand. Concurrently 7.5 ml of substrate was added to Compartment I. The surface radioactivity showed that while in Compartment I the surface concentration remained unchanged, there was practically no adsorption of the enzyme in the presence of substrate in Compartment IV. The enzymatic activity of the solutions in Compartment I where a surface layer was allowed to be formed before the addition of the substrate was always lower than the enzymatic activity in the reference solution in Compartment IV.

The enzymatic activity in Compartment I $(e.a.)_I$ was, however, higher than in the removed subphase $(e.a.)_S$. The contribution of the surface layer to enzymatic activity is $(e.a.)_I - (e.a.)_S$. The loss of enzymatic activity in the solution due to adsorption is noice $(e.a.)_{IV} - (e.a.)_S$. From here the fraction of the retained enzymic activity in the surface layer is

$$\xi = \frac{(e.a.)_I - (e.a.)_S}{(e.a.)_{IV} - (e.a.)_S} \tag{7}$$

In Fig. 7 ξ is plotted against the surface concentration at the air–water interface. The amount adsorbed at the air–water interface was about 75% of the total enzyme lost from the solution due to adsorption, as determined from the enzyme concentration in Compartment IV and in the removed subphase. The retained fraction of enzymatic activity increases with surface concentration. The dependence could not be accurately

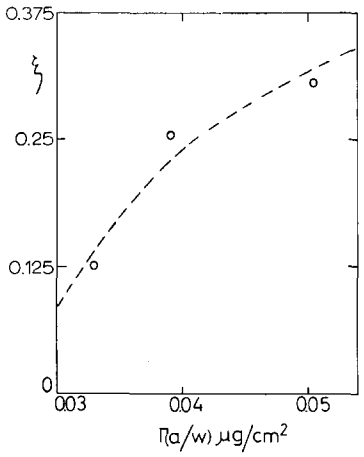

Fig. 7. Fraction of the retained enzymatic activity in the adsorbed surface layer ξ of trypsin as a function of its surface concentration at the air–water interface Γ(a/w).

determined, since ξ could be determined accurately enough within the surface concentration region given in Fig. 7. This corresponds to the bulk concentrations between 0.4 and 1 μg/ml.

ξ is related to the activity of the enzyme adsorbed both at the air–water interface and on the stainless steel. Since the adsorption on the stainless steel is weaker than at the air–water interface, it may be assumed that the enzyme on the stainless steel retains a larger fraction of its enzymatic activity. If there would be no loss in activity on the stainless steel, then the calculated fraction of the enzymatic activity retained at the air–water interface would be close to zero at 0.03 μg/cm^2 and about 0.35 at 0.05 μg/cm^2.

Enzymatic activity of monolayer spread with the aid of isopropanol

Isopropanol enables the incorporation of relatively large quantities of trypsin into the surface layer which makes the determination of the low enzymatic activity possible. After spreading the monolayer and successive washing, half of the subphase was transferred from Compartment I to IV. At this point two portions of a solution of 0.2 mg/ml Bz-Arg-ONAn equal in volume to half of the substrate were injected underneath the monolayer in Compartment I and added to Compartment IV. Both compartments were stirred gently so as not to disrupt the surface layer and the change in absorbance at $\lambda = 400$ nm as a function of time was measured. The enzymatic activity measured in Compartment IV was taken as the blank and was removed from the activity measured in Compartment I to obtain the net activity of the surface layer. Knowing the concentration of the enzyme remaining in the subphase, its specific

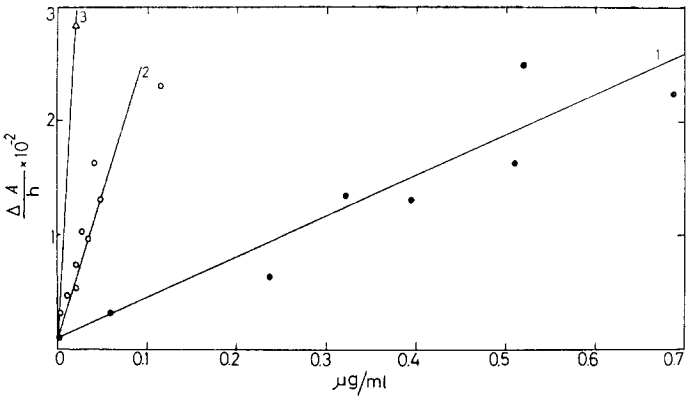

Fig. 8. Enzymatic activity of trypsin at 30 °C expressed in $A_{400\ nm}$/h as a function of the quantity of enzyme per ml of substrate solution. 1, in the surface layer; 2, in the subphase; 3, in the intact solution.

activity could also be obtained and is presented in Figs 8 and 9 together with the activity of the intact enzyme in solution and of the surface layers. As seen from Figs 8 and 9 the activity of the enzyme in the monolayer at 30 °C is about 3% of its activity in the bulk while at 20 °C it is less than 2% of the bulk activity. On the other hand, the solution in contact with the monolayer seems to contain more inactivated enzymes at 30 than at 20 °C. The activity of the enzyme in the subphase at 20 °C is about 40% of the intact enzyme while at 30 °C it is only about 25% of the original enzyme.

The substrate caused a partial dissolution of the enzyme from the monolayer. Comparing the reduction in counts from the surface Δ cpm with the increment in bulk concentration as determined by scintillation counting provided another means

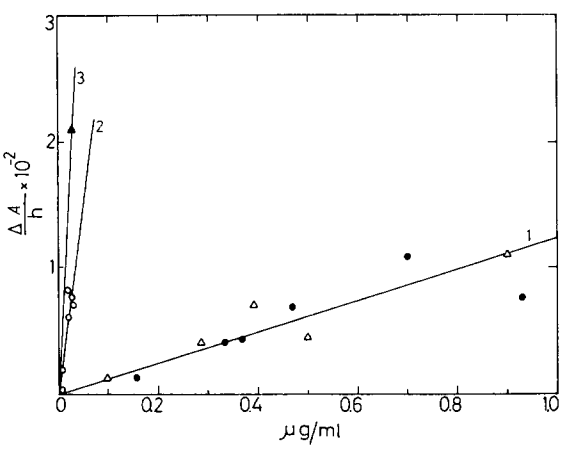

Fig. 9. Enzymatic activity of trypsin at 20 °C expressed in $A_{400\ nm}$/h as a function of the quantity of enzyme per ml of substrate solution. 1, in the surface layer; 2, in the subphase; 3, in the intact solution.

for the determination of the surface concentration from the measured radioactivity from the surface. The surface concentration Γ_p is then given by

$$\Gamma_p = \frac{(\text{cpm})_\sigma}{\Delta(\text{cpm})_\sigma} \Delta\Gamma_p$$

where $\Delta\Gamma_p$ is the trypsin dissolved under the influence of the substrate from the surface as determined by scintillation counting. The good agreement between the surface concentrations determined by this method and by the calibration of the surface counter by monolayers of tritium-labelled oleic acid is further evidence that acetylation did not appreciably change the surface activity of the trypsin.

DISCUSSION

Adsorption of enzymes at interfaces is a complex process, during which there may be a conformational change. All the evidence indicates [1, 10, 11] that the secondary structure is little affected by the adsorption forces while there is a large effect on the tertiary structure and the biological activity. Depending on the adsorption forces the protein may be adsorbed reversibly or irreversibly. The reversibility or irreversibility of adsorption may be defined only in relative terms with respect to the experimental conditions. A substance is considered to be irreversibly adsorbed if it cannot be desorbed by reducing the concentration within "a reasonable time". Thermodynamically, irreversible adsorption is characterized by a lack of equilibrium between the substance in the bulk phase and in the surface phase. The chemical potential of an irreversibly adsorbed substance varies independently from the variations in the bulk phase. The lack of equilibrium means that there is a potential barrier between the substance in the bulk phase and in the adsorbed state. In the case of an enzyme, a different state also means a difference in enzymatic activity. Some enzymes are more active in the interfacial state when incorporated in biological membranes. Trypsin is inactivated at the air–water interface.

The degree of inactivation increases with time, with reduced surface concentration and with added spreading agent, e.g. isopropanol. A completely surface inactivated trypsin molecule remains inactive even after displacement from the surface. We were not able to observe any reactivation within the time of our experiments, which amounted to many hours. The irreversible adsorption induces irreversible conformational changes of an as yet unknown chemical nature. The conformational change may also facilitate interaction between the molecules initiating aggregation in the surface layer. Slow aggregation occurring in the bulk phase at pH 4.8 [13] may also be preceded by a slow conformational change.

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