POLAROGRAPHIC BEHAVIOUR OF PROTEINS WITH MODIFIED HISTIDINE RESIDUES

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In the Brdička catalytic polarographic reaction of proteins it is assumed that the presence of cysteine or cystine residues in the protein molecule is essential for the effect. The role of other amino acid residues, however, is not clear. The authors found that if histidine residues are specifically modified, the catalytic effect of the protein is significantly lowered. A tentative explanation of the role of histidine in the catalytic process is offered.

When proteins and peptides are studied by classical polarography they exhibit a special feature: in buffered solutions of Co^{2+} or Co^{3+} salts they produce a characteristic double wave, discovered first by Brdička[1,2]. Since then it has been established[1,3,4] that it is the presence of SH or S—S groups in the protein molecule that is necessary for the catalytic effect. Today it is assumed[1] that a complex of Co^{2+} with SH groups and possibly some other functional groups incorporated in the protein molecule is formed as an essential step in the catalytic process. Proteins, being surface active substances, adsorb on the electrode surface which, according to many authors[5,6], is also very important in the catalytic process. Many authors have proposed various hypotheses to explain the double wave character of the catalytic effect: some ascribe different roles to different parts of the protein molecule [7–9], others[5,6,10–13] suggest that the two waves are due to different adsorption properties of the protein during the potential range of the polarographic effect. Nevertheless, the actual origin of the double wave remains unresolved to this day.

Histidine is, from the polarographist's point of view, a very intriguing amino acid: it should form a cobalt complex very easily and it exhibits a single peaked wave in the potential range of −1·5 to −1·6 V *vs* saturated calomel electrode (S.C.E.), though this effect is much smaller than that of cystein. However, it has been studied very rarely: Shinagawa and coworkers[14] conclude that the maximum is caused mainly, though not entirely, by streaming of the electrolyte in the neighbourhood of the electrode. Gudbjarnason[15], on the other hand, regards the effect as a catalytic one and even found a double wave at lower pH.

In this study we set out to elucidate the role of histidine in the catalytic polarographic effect of proteins. We chose four well defined proteins whose three-dimensional structure is known, as our model proteins: insulin, lysozyme, ribonuclease A and α-chymotrypsin.

All these proteins are polarographically active; of the three S—S bonds in the insulin molecule two are reducible at the electrode and only these two are catalytically active[16,17]; both histidine residues are on the surface of the molecule[18]. In lysozyme it is assumed that all four S—S bonds

take part in the polarographic reaction[19] and the only histidine residue is accessible from the surface of the molecule[20]. The situation in ribonuclease A is similar to that of lysozyme where the S—S bonds are concerned; of the four histidine residues three are located in the enzyme active centre, while the fourth is apparently in a different part of the molecule[21]. Of the five S—S bonds in α-chymotrypsin one is polarographically "buried", the other four are active[19]. One of the two histidine residues is part of the enzyme active centre while the other is somewhat removed and out of contact with this centre[22].

EXPERIMENTAL

The polarographic experiments were carried out on a PO 4 polarograph (Radiometer, Copenhagen) in a Kalousek vessel with S.C.E. as the reference electrode. The dropping mercury electrode characteristics in the Brdička solution were (at $-1{\cdot}5$ V *vs* S.C.E.): $t_1 = 1{\cdot}9$ s; $m = 4{\cdot}1$ mg/s for the first and $t_1 = 2{\cdot}0$ s; $m = 3{\cdot}8$ mg/s for the second electrode. The composition of the supporting electrolyte, the Brdička solution, was: 1.10^{-3}M-$CoCl_2$, 0·1M-NH_4Cl, 0·1M-NH_4OH. This solution was freshly prepared before each set of expeirments. The wave heights were measured taking the limiting diffusion current of cobalt as the base line. The concentration of the protein solutions, of which 0·1 ml was pipetted into 10 ml of the supporting electrolyte, was as follows: insulin (Léčiva, Prague) 5.10^{-4}M; lysozyme (hen's egg-white, Serva) 6.10^{-5}M; ribonuclease A (bovine pancreatic, Serva) 8.10^{-5}M; α-chymotrypsin (bovine, Serva) 8.10^{-5}M. The proteins were used without further purification. The histidine-modifying reagent, diazonium-1-H-tetrazole (DHT), was prepared from 5-amino-1*H*-tetrazole (Schuchardt, München) and used according to Suzuki and coworkers[23].

Spectrophotometric measurements were performed on a Universal spectrophotometer VSU-1 (Zeiss, Jena) with a NaCl prism at 480 nm. Solutions of DHT-modified 10^{-5}M insulin, $1{\cdot}2 . 10^{-5}$M lysozyme and $1{\cdot}6 . 10^{-5}$M ribonuclease and α-chymotrypsin were measured against buffer and DHT solutions as blanks. Circular dichroism spectra were measured on a Dichrograph II model 185 made by Roussel-Jouan, Paris.

Electrocapillary curves were measured by determining the dependence of the drop-time on the applied potential for solutions of both native and DHT-modified proteins.

RESULTS

Determination of Wave Heights

The determination of wave heights becomes a complicated problem when the protein double wave is measured. This wave occurs either as a "classical" double wave with two distinguishable peaks or as one rounded maximum, but very often as something between these two shapes, according to the conditions of the solution. Fortunately in most cases only the height of the double wave as a whole is needed and then conditions are used which induce one peak. In this study, however, we wanted to measure both waves of the double wave separately. The supporting electrolyte used did separate the two waves of all four proteins in their unmodified state. Then, after the proteins had reacted with DHT, the two waves usually merged into one round maximum with a "shoulder" at more negative potentials. The curves that resulted with growing DHT concentration all seemed to indicate that the original double wave really remains

as such but the height of each of its waves alters to a varying degree. We therefore applied a method similar to a common practice in spectrophotometry: we dissolved each double wave into two symmetrical peaks (Fig. 1). Two premises were used: *a*) the double wave results on superimposing two symmetrical peaks; *b*) the potential of the first wave remains constant throughout a series of measurements.

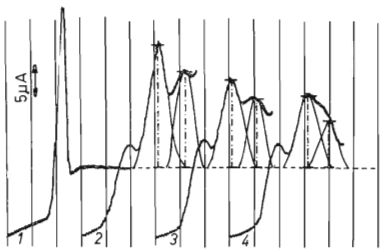

FIG. 1

Examples of the Determination of Wave Heights

1 Supporting electrolyte ($Co^{2+} \rightarrow Co^0$ reduction wave). *2*, *3*, *4*: different protein double waves, dissolved into two symmetrical maxima each. Full supposed shape of the symmetrical maxima; dashed base line (cobalt limiting diffusion current); dash and dot symmetry axis of the maxima.

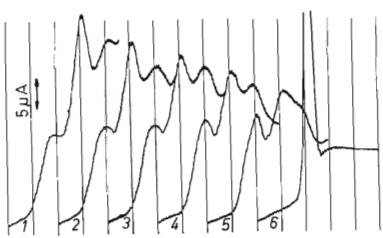

FIG. 2

Polarograms of Native and Modified Insulin

0·1 ml of 5.10^{-4}M insulin solution in 10 ml of supporting electrolyte, modified by: *1* 0; *2* 2·8; *3* 5·6; *4* 8·4; *5* 14 mM-DHT. *6* supporting electrolyte. From −0·8 V (S.C.E.), 0·2 V/absc., capillary No 1.

We found that this method, though time consuming, gives satisfactory results and permits the determination of the heights of both waves even where they have seemingly merged. The potential of the peak of the second wave then shifts very slightly, by 0·06 V at the most, through one series. We think that this empirical method, elaborated in greater detail, could be widely used for problems in which two polarographic peaks in close contact are to be measured separately.

Modification of Proteins

When histidine as a free amino acid is modified with DHT its polarographic maximum disappears completely. When the four proteins are used their reaction with DHT has a marked effect on their polarographic behaviour: the typical double wave changes its height and its shape as well (Fig. 2). Fig. 3 shows the dependence of wave heights and absorption at 480 nm on the concentration of DHT for all four proteins. The first polarographic wave diminishes by approximately 45%, the second by 50% of the initial wave height in all cases except lysozyme (first wave by 37%, second by 39%).

The circular dichroism spectra of native and DHT modified insulin and ribonuclease respectively are very similar in the range of 260–200 nm, indicating practically no changes of conformation induced by reaction with DHT. Solutions of α-chymotrypsin which is a practically non-helical protein had to be measured in larger cuvettes to obtain a measurable CD signal. The absorption of the intensively coloured DHT derivative of α-chymotrypsin was then unfortunately too great to permit any serious conformational analysis. To elucidate the effect of DHT modification on the polarographic reaction of proteins we measured the electrocapillary curves of all systems involved. We found that when the histidine residues in a protein molecule are chemically modified the surface activity of the protein on the mercury electrode is lowered.

DISCUSSION

The most important facts which follow directly from the experimental results are: *a*) Both waves of the double wave are lowered to a similar extent by the reaction of proteins with DHT. On subjecting histidine residues of insulin to modification by bromoacetone[24] we found[25] that the kinetics of this reaction determined polarographically are the same for both waves. We therefore assume that the catalytic double wave is affected very similarly throughout the whole of its potential range by chemically modified histidine residues. *b*) The surface activity (solution–mercury interface) of the protein is lowered after DHT modification of the histidine residues. *c*) Fig. 3 illustrates an interesting phenomenon: while the curves representing the dependence of wave height on DHT concentration have a similar shape for all four proteins, the spectrophotometric curves vary from protein to protein. The lysozyme curve (one histidine residue) and insulin curve (two histidine residues, both accessible

for the reagent[18,23]) are just as simple in shape as the corresponding wave height dependence curves, but the ribonuclease and α-chymotrypsin spectrophotometric curves both exhibit a "plateau" which appears at a DHT concentration sufficient to achieve the limiting polarographic wave height. Then the spectrophotometric curve rises further to a value of absorption which corresponds to total histidine content for both proteins. Now these two steps on the spectrophotometric curve indicate two degrees of accessibility of histidine residues in this reaction[23] – incidentally in both cases the first "plateau" corresponds to the modification of one histidine residue. From these facts it is reasonable to assume that only the modification of histidine

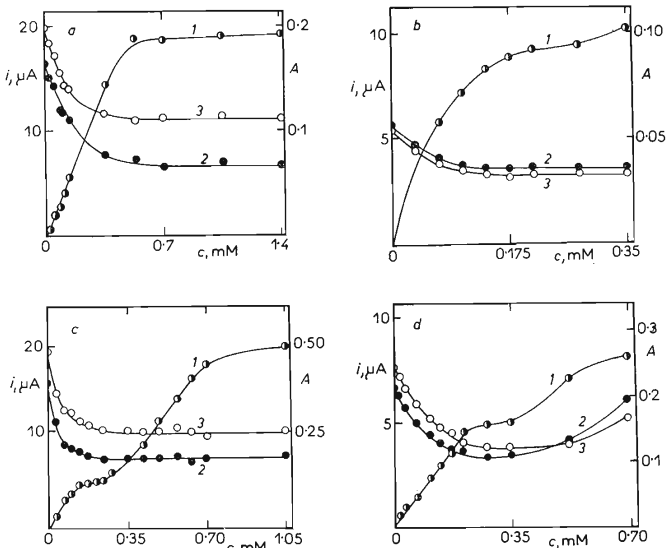

FIG. 3

Spectrophotometry and Polarography of Native and Modified Proteins

◑ Absorbancy at 480 nm (*A*); ○ height of first polarographic wave(*i*); ● height of the second polarographic wave (*i*). Coordinates: concentration of DHT in the reaction mixtures. *a* insulin; *b* lysozyme; *c* ribonuclease A; *d* α-chymotrypsin (see text for protein concentrations).

residues most easily accessible ("surface histidines") in a given protein brings about marked changes in their polarographic behaviour. *d*) The diminished polarographic activity of DHT modified proteins is not accompanied by any gross destruction of their native conformation as can be seen from CD measurements.

There seem to be two possible explanations for the way in which the histidine residue could take part in the catalytic process of proteins: *1*) The histidine residue itself is responsible for the polarographic effect of proteins, not cystine as has hitherto been assumed. However, there is evidence against this concept. The catalytic activity of histidine as a free amino acid is relatively low[14] (about a hundred times smaller than that of cysteine). While peptides and proteins that do not contain any SH or S–S groups are, as far as is known, not catalytically active, those peptides and proteins which do include cysteine (cystine) in the molecule are catalytically active without necessarily having any histidine residues as part of the molecule[8] (oxytocin, vasopressin). *2*) The catalytic effect is caused, as is commonly understood, by SH groups in proteins (or SH groups generated on the electrode by the reduction of S–S bonds) and the histidine residues in the molecule cooperate in some way to give the resulting catalytic effect. The second alternative appears to be more probable, in view of our results, as the following paragraphs will show.

Cobaltous ions, in a solution containing proteins, tend to form complexes with various ligands, either SH groups or basic and carboxylic groups of amino acid side-chains. Therefore, in solutions of proteins that do not contain free SH groups (which is the case of our four proteins) the only possible ligands are these other groups and especially the imidazole group of histidine. Of course, under conditions of the catalytic effect (*i.e.* applied voltage) the S–S bonds in the vicinity of the electrode are reduced to SH groups and these in their turn are capable of forming cobalt complexes. It has been shown that among low-molecular compounds the highest catalytic activity is found particularly in five-membered chelate rings with Co^{2+} as the centre ion[26,27] and it seems that these remain in existence after the reduction of Co^{2+} to Co^0 on the electrode.

In the case of our four proteins, of all possible ligands mainly the imidazole rings of histidine are modified by DHT. We realized, of course, that DHT does also react with tyrosine residues. However, to eliminate doubts about the possible effect of modified tyrosine residues on the double wave, we carried out a reaction of lysozyme and α-chymotrypsin with N-acetylimidazole[28] which is assumed to be a specific tyrosine reagent. No change of the polarographic effect was observed. We therefore assume that all changes in the polarographic behaviour of proteins in our experiments are due solely to the changed properties of histidine residues.

We presume that the original cobalt complex (that contained imidazole ligands) will be changed to one with no imidazole groups. This new complex may be less advantageous for the formation of the assumed catalytically active cobalt complex. At the

same time, though, we found that the modification of histidine residues of a protein results in lowered surface activity of its molecule. The stabilizing effect of adsorption on the catalytically active cobalt complex is therefore lower and the catalytic activity of the protein diminishes.

In view of our results we should like to emphasize that from now on, when trying to explain the Brdička catalytic effect of proteins, polarographists will have to take the role of histidine residues into consideration.

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