POLAROGRAPHY OF PROTEINS IN Co(II)-AMMONIA BUFFER SOLUTION

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The polarographic catalytic activity of ten proteins has been studied in presence of cobaltous--ammonia buffer solution using a dropping mercury electrode. For the proteins investigated the $B(Co^{2+})$ constant, which characterises the polarographic protein activity, was determined. Using the total number of half-cystine residues and the number of chemically accessible half-cystine residues the polarographic catalytic increments were calculated. These increments can be used for calculation of polarographically accessible half-cystine residues.

Polarographic protein activity can be characterised by a constant B, defined as the current in microamperes per square millimeter of the electrode surface at one micromolar concentration of the protein[1]. As the polarographic protein double wave is related to the number of disulphide and sulphydryl groups of cystine and cysteine in the molecule[2-4], the value of the constant number can provide information regarding the content of these groups in the protein molecule and their behaviour.

EXPERIMENTAL

Ten proteins were studied in presence of cobaltous ammonia buffer solution using a Radiometer PO 4 polarograph, a dropping mercury electrode and a Kalousek cell with S.C.E. The composition of the supporting electrolyte was 0·1M-NH_4Cl, 0·1M-NH_4OH and 0·001M-$CoCl_2$. The capillary constants were: $m = 2{\cdot}326$ mg/s, $t = 1{\cdot}98$ s at $-1{\cdot}53$ V and the mean surface area $P = 0{\cdot}51m^{2/3}t^{2/3}$ was found to be 1·41 mm^2. All readings were recorded from $-0{\cdot}80$ V to $-1{\cdot}70$ V *vs* S.C.E. in an atmosphere of nitrogen at a temperature of 22 ± 1°C.

Lysozyme, ribonuclease, β-lactoglobulin, chymotrypsin, trypsin and pepsin were from Serva (Federal Republic of Germany), insulin was a Zn-salt prepared by Organofarma (Prague, Czechoslovakia), human serum albumin was from Sevac (Michalany, Czechoslovakia), orosomucoid was prepared in this laboratory from fraction VI using cellulose ion exchangers, and ovalbumin was prepared in the biochemical laboratories of this Department. The amino acid cystine was from Lachema (Brno, Czechoslovakia).

The proteins were dissolved in redistilled water to prepare the stock solution ($1 \cdot 10^{-4}$M), insulin being dissolved in the pH 8·5 bicarbonate buffer. Each protein was studied in the concentration range $2{\cdot}5 \cdot 10^{-7}$ to $3{\cdot}0 \cdot 10^{-6}$M. For registration of the reference line corresponding

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to the height of the cobalt wave 0·5 ml of 0·5% solution of gelatine was added to 10 ml of the supporting electrolyte to suppress the cobalt maximum. The protein waves were read off at the potential of the second maximum of the double wave.

Calibration curves were drawn for each of the ten proteins investigated from an average of 8 to 10 sets of experiments and the standard deviations were calculated. The polarographic constants B (Co^{2+}) for the proteins were determined at 1 micromolar concentration, from plots of the current density i_K/P in $\mu A/mm^2$ against the protein concentration in micromoles. Results obtained were then compared with the calibration curve plotted for cystine. Glutathione was found to be unsuitable as calibrating agent for this low concentration range.

RESULTS

The catalytic double waves observed for the different proteins at 1 micromolar concentration are shown in Fig. 1. Fig. 2 shows the concentration dependence of the catalytic current density in $\mu A/mm^2$ for these proteins, together with the standard deviations. It is found, in general, that a nearly linear relationship is followed at lower concentrations but the curves tend to approach a limiting value with increasing concentration, especially in the cases of human serum albumin and ovalbumin.

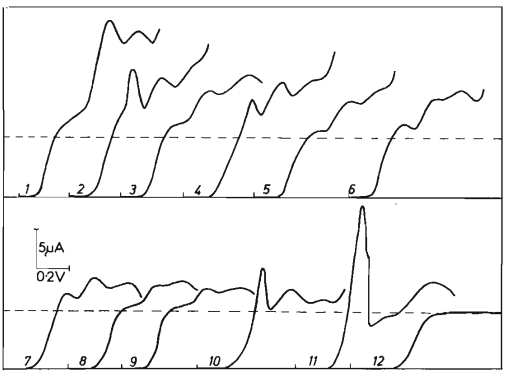

FIG. 1

The Catalytic Double Waves of the Proteins and of Cystine at 1 Micromolar Concentration

1 Human serum albumin; *2* lysozyme; *3* β-lactoglobuline; *4* ribonuclease; *5* trypsin; *6* chymotrypsin; *7* pepsin; *8* ovalbumin; *9* orosomucoid; *10* insulin; *11* cystine; *12* supporting electrolyte + + gelatine.

The values of the $B\,(Co^{2+})$ constants determined at 1 micromolar concentration, together with the half-cystine contents of the proteins are presented in Table I.

DISCUSSION

From Table I we find that there is a non-linear relationship between the $B\,(Co^{2+})$ values of the proteins and their half-cystine content. Thus lysozyme and ribonuclease, having 8 half-cystine residues each, show higher $B\,(Co^{2+})$ values compared to that of trypsin or chymotrypsin which have a half-cystine content of 12 and 10, respectively. The nonlinearity is reflected in the values of the ratio of the $B\,(Co^{2+})$ constant to the half-cystine content of the protein. This ratio may be defined as the polarographic catalytic increment[5] showing the catalytic activity per one half-cystine residue (Table I). An explanation for the differences in the catalytic increment from one protein to another can be found in the accessibility of the disulphide or sulphydryl groups.

Many authors have observed that the proteins possess different disulphide and sulphydryl groups from the point of view of their accessibility ("free and buried" groups). The number of accessible groups determined depends much on the reducing

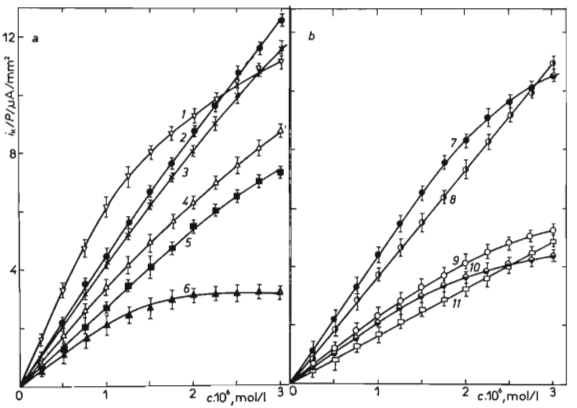

FIG. 2

The Concentration Dependence of the Catalytic Current for the Ten Proteins and Cystine

a: *1* Human serum albumin; *2* lysozyme; *3* ribonuclease; *4* chymotrypsin; *5* cystine; *6* ovalbumin; *b*: *7* β-lactoglobulin; *8* trypsin; *9* pepsin; *10* orosomucoid; *11* insulin.

agent, the method of reduction and the presence of denaturing agents[6]. In our case, a relatively suitable approach seems to consists in considering the number of disulphide groups which have been found to be accessible by the chemical reduction of proteins in absence of any denaturing agent. The number of such groups for the different proteins are shown in Table I. An alternative approach would be to consider the number of electroreducible disulphide groups obtained by Cecil and Weizman[7]. These authors studied the reducibility of the disulphide groups of five proteins using the dropping mercury electrode mostly at pH 1. However, as our experiments were performed at pH 9·3 and in the presence of Co(II)ions, the observations by the above authors were not quite suitable for our considerations, except for insulin

TABLE I

Polarographic Constant B (Co^{2+}) and Polarographic Catalytic Increments of Ten Proteins and Their Half-Cystine Content

Protein	Total No of 1/2 cystine residues	$B(Co^{2+})$	$\frac{B(Co^{2+})}{\text{total 1/2 cystine}}$	Accessible 1/2 cystine residues	„Corrected" increment
Human serum albumin	33	61·5	0·19	3[a]	2·05
β-Lactoglobulin	19	4·32	0·23	2[b,c]	2·16
Trypsin	12	3·64	0·30	4[d,e]	0·91
Chymotrypsin	10	3·43	0·34	4[e]	0·86
Lysozym	8	4·50	0·56	4[f]	1·12
Ribonuclease	8	4·31	0·54	4[e,g]	1·08
Ovalbumin	6—7	2·13	0·35—0·30	3[h,i]	0·71
Pepsin	6	2·28	0·38	4[j,k]	0·57
Orosomucoid	6	2·09	0·35	—	—
Insulin	6	1·69	0·28	4[l,m]; 2[n]	0·42; 0·84
Cystine	2	2·71	1·35	2	1·35

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which was reduced at pH 7·2 (2 disulphide groups were reducible). The value of 1·4 half-cystine residues at pH 9·2 for ribonuclease would give an exceptionally high polarographic catalytic increment as compared to that of cystine, and therefore the chemical value of 4 half-cystine residues has been prefered for this protein[8,9].

The polarographic increments calculated with respect to the number of accessible disulphide groups may be termed as the "corrected" increments. These values are shown in the last column of Table I. Four proteins (trypsin, chymotrypsin, lysozyme and ribonuclease) are found to have such "corrected" increment values which are closer to one another than in the previous case of "incorrect" increments. The approximately one-third lower values than that of cystine can probably be attributed to the lower activity of disulphide and sulphydryl groups built in these large protein molecules in comparison with the much smaller, cystine molecule. Also other factors as diffusion coefficient, rate of recombination of the SH group, *etc.* can play a certain role in these catalytic processes.

The corrected polarographic increments for serum albumin and β-lactoglobulin are higher than those of other proteins and of cystine. The reason for this is either in the low value of the chosen accessible half-cystine residue (*i.e.* additional disulphide groups and polarographically accessible), or the free disulphide and sulphydryl groups of the proteins have greater polarographic activity than that of cystine. Considering the first assumption as more probable, then for serum albumin one sulphydryl group (corresponding to mercaptalbumin) and two or three disulphide groups (*i.e.* 5 ot 7 half-cystines) are polarographically active while for β-lactoglobulin it is 2 sulphydryl groups and 1 or 2 disulphide groups (*i.e.* 4 or 6 half-cystines). As for pepsin and ovalbumin, the reason for the relatively low polarographic increment values is not yet known. Insulin, with a comparatively low molecular weight and a high-cystine content shows a surprisingly low increment of 0·42. This value would be more acceptable considering the number of accessible half-cystine residues to be 2 instead of 4 as shown by Fraenkel-Conrat and Fraenkel-Conrat[10] from experiments reduction using thioglycolate. In spite of this, however, the corrected increment will be 0·84 which is somewhat lower than the values obtained for the four proteins mentioned previously.

The catalytic polarographic increment is a physical constant of a pure native protein, characterising the protein from the point of view of the number of accessible disulphide and sulphydryl groups. From a knowledge of the total number of the half-cystine residues from chemical amino acids analysis, the number of accessible disulphide and sulphydryl groups of a protein can be approximately estimated. For proteins having a half-cystine content of 8 to 12 and with molecular weight between 10000 and 30000 the mean corrected increment was found to be 0·99. The number of accessible half-cystine residues of a protein may then be calculated from the ratio of the polarographic constant to the mean increment.

This idea may be applied to the case of orosomucoid. Although the structure of orosomucoid has been intensively studied not much is known about the accessibility of its disulphide groups. Taking the mean increment as 0·99 the number of accessible half-cystine residues is 2·09/0·99 *i.e.* 2·11. Polarographic data suggest that one disulphide group of orosomucoid is more accessible than the other two. This concept must be further examined, however, with a larger number of defined proteins having different halfcystine residues and molecular weights.

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