BBA 4040

A POLAROGRAPHIC STUDY OF CHROMIUM–INSULIN–MITOCHONDRIAL INTERACTION

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(Received November 21st, 1962)

SUMMARY

1. Insulin exhibits a polarographic wave due to the reduction of a disulfide group. This wave most probably arises from the reduction of the intrachain disulfide.

2. Chromium(III) forms complexes with insulin and mitochondria, both complexes involving chromium–sulfur linkages.

3. Insulin reacts with mitochondria through the formation of a sulfhydryl–disulfide linkage. This reaction is enhanced by the presence of chromium(III).

INTRODUCTION

Chromium(III) has been identified as the active ingredient of the glucose-tolerance factor, a dietary agent required for maintenance of normal glucose tolerance in the rat[1]. Deficiency of this factor leads to an impairment of glucose-removal rates *in vivo* and to an impaired response of epididymal fat tissue to small concentrations of insulin *in vitro*. Both conditions can be restored to normal with trace amounts of chromium; for this effect, small, near-physiological amounts of insulin are indispensable[2]. Furthermore, it was shown that chromium exerts the same stimulation of the action of insulin on transport rates of nonutilizable sugar, thus locating its main site of action on the entry mechanism of the cell membrane[15].

These findings suggested a close relation between chromium and insulin; the possibility of such a relation was investigated in the study presented here. Since intact tissue is not suited for polarographic measurements, rat-liver mitochondria were used for these experiments. A direct effect of the hormone on mitochondrial swelling in the presence of sulfhydryl compounds was recently demonstrated[3].

EXPERIMENTAL

Reagent-grade chemicals were used with the exception of sucrose which was purified by deionization to remove all trace metals. Amorphous insulin, Lot W-3255, was obtained from Eli Lilly and Co. A 60% suspension of rat-liver mitochondria in 0.25 M deionized sucrose was prepared by the method of NEUBERT AND LEHNINGER[3].

Abbreviation: SCE, saturated calomel electrode.

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All polarographic measurements were made with a Sargent Model-XV Polarograph. A Lingane H-cell fitted with a 3% agar–satd. KCl salt bridge and a satd. calomel reference electrode was employed. The solution under study was placed in the sample compartment of the H-cell and flushed with N_2 for 10 min prior to each run. During the course of the polarogram, a N_2 atmosphere was maintained over the sample solution. "Seaford" N_2 (Southern Oxygen Co.) was used throughout the investigation. All polarograms were obtained on a sample thermostatted at 23° ± 0.2°.

Reversibility of the electrode reactions was tested by determining the slopes of the $\log(i_d - i)/i$ *vs.* E plots. From these slopes may be calculated the "n-value", the number of electrons involved in the reversible electrode reaction. Non-integer values of n indicate an irreversible electrode reaction. Values for the half-wave potentials, $E_{1/2}$, were taken from these logarithmic plots. Data for these plots were obtained by manual operation of the polarograph. Applied voltages were determined by measurement with a student potentiometer.

The apparent instability constant of the chromium(III)–insulin complex was determined by the method described in KOLTHOFF AND LINGANE[4].

DISCUSSION OF RESULTS

In a previous paper the authors described a polarographic wave for mitochondrial suspensions in 1 M KNO_3 supporting electrolyte[5]. This wave had a half-wave potential of approx. −0.290 V *vs.* SCE and an n-value of 0.6–0.7. In fresh mitochondrial preparations this wave was attributed to sulfhydryl groups contained in the protein structure of the mitochondrial membrane.

It has been reported that insulin in BRDICKA's solution[6] (10^{-3} M $CoCl_2$, 0.1 M NH_4OH, 0.1 M NH_4Cl) gives the catalytic double-wave characteristic of proteins[7]. Since the double wave is caused by the presence of a sulfhydryl group[8] and since insulin contains no sulfhydryl groups, the protein double wave must arise from sulfhydryl groups produced by the reduction of disulfide linkages. This reduction takes place at potentials more positive than the protein double wave[9].

In the present study, insulin, in several supporting electrolytes not including BRDICKA's solution, has been found to exhibit as many as three waves. In an orthophosphate buffer (pH 2.5, I 0.2) a 10^{-4} M solution of insulin gave a single wave with a half-wave potential of −0.328 V *vs.* SCE and an n-value of 0.48. $2 \cdot 10^{-4}$ M insulin in 1 M KNO_3 (pH 5.3) exhibited two waves with half-wave potential values of −0.504 V ($n = 0.56$) and −1.088 V ($n = 0.94$), respectively. Three waves were obtained for 10^{-4} M insulin in orthophosphate buffer (pH 7.4): $E_{1/2} = -0.124$ V, $n = 0.38$; $E_{1/2} = -0.643$ V, $n = 0.5$; and $E_{1/2} = -1.102$ V, $n = 0.24$. The orthophosphate buffers employed in these studies were prepared according to the directions of CHRISTIAN AND PURDY[10].

The above data indicated that the wave for insulin at pH 2.5, the first wave at pH 5.3, and the second wave at pH 7.4 were all due to the reduction of a disulfide group of insulin to a sulfhydryl group. CHEVALIER AND PURDY[11] have found that the reduction of a disulfide group took place at a half-wave potential of about −0.700 V *vs.* SCE, $n = 0.5$. This study was made in an orthophosphate supporting electrolyte of pH 7.4. The three waves for insulin cited above have essentially the same n-value and in addition, the half-wave potentials follow the logarithmic relation-

ship between hydrogen-ion concentration and potential is defined by the Nernst equation expression for the reaction:

$$RSSR + 2H^+ + 2e \leftrightharpoons 2RSH \qquad (1)$$

Furthermore, the potential of the insulin disulfide wave in pH-7.4 supporting electrolyte is more positive than that obtained with ordinary disulfide groups, indicating that the insulin disulfide is more easily reduced. This would suggest that the insulin wave arises from the reduction of the intrachain disulfide. The work of LINDLEY[12] supports this observation.

Complexes formed with chromium (III)

In 1 M KCl supporting electrolyte (adjusted to pH 7.4 by the addition of KOH), 10^{-4} M chromium (III) gave a wave for the reduction of chromium (III) to chromium-(II). The change of electrolyte was necessitated by differences in polarographic response of chromium (III). In 1 M KNO_3 medium no satisfactory reproducible wave could be obtained for the reduction of chromium (III). Upon the addition of insulin to a solution of chromium (III) in 1 M KCl, the chromium wave shifted to more negative potentials, indicating complex formation. The magnitude of this shift depends upon the stability of the complex formed, the concentration of the complexing agent, and p, the number of moles of complexing agent reacting with 1 mole of the cation. Examination of the polarographic data indicated that 3 insulin molecules were associated with each chromium (III) ion and that the complex formed had an apparent instability constant of $10^{-14.5}$; the active site is most probably the intrachain disulfide of the insulin molecule. No evidence of complex formation between chromium (III) and insulin was found in solutions of pH 5.3 or 2.5.

The addition of mitochondrial suspension to an electrolyte containing 10^{-4} M chromium (III) caused the chromium wave to shift to more negative potentials. This shift indicated the formation of a complex between chromium (III) and mitochondria; the active site was the free sulfhydryl groups of the mitochondrial membrane. Complexes were also formed between mitochondria and copper (II), zinc, and cobalt (II).

The interaction between insulin and mitochondria

To 1 ml of a 60% suspension of mitochondria (in 0.25 M deionized sucrose) and 5 ml of 1 M KNO_3 supporting electrolyte was added insulin solution to a final concentration of $5 \cdot 10^{-5}$ M. The addition of insulin caused the mitochondrial wave to shift to more positive potentials; this shift was not accompanied by a change in slope of the mitochondrial wave. At final insulin concentration of $5 \cdot 10^{-4}$ M, the shift of the mitochondrial wave amounted to 15 mV. On the other hand, when mitochondrial suspension was added to a 10^{-4} M insulin solution, there was no noticeable shift in the insulin wave.

To 1 ml of a 60% suspension of mitochondria (in 0.25 M deionized sucrose) and 5 ml of 1 M KNO_3 supporting electrolyte was added chromium (III) solution to a final concentration of 10^{-3} M. No shift in the mitochondrial wave was noted with chromium (III) concentrations less than 10^{-3} M. However, when the chromium (III) concentration was increased above 10^{-3} M, the mitochondrial wave shifted to more positive potentials. At a final chromium (III) concentration of $3 \cdot 10^{-3}$ M, the shift of the mitochondrial wave amounted to 60 mV. This shift of the mitochondrial wave was accompanied by a change in slope as the n-value approached unity.

Finally, chromium(III) solution was added to a sample consisting of 1 ml of mitochondrial suspension and $5 \cdot 10^{-5}$ M insulin. The addition of chromium(III) solution to a final concentration of 10^{-3} M caused the mitochondrial wave to shift to more positive potentials; the magnitude of the shift was 100 mV, approx. 3-fold greater than the sum of the shifts caused by these concentrations of chromium(III) or insulin alone.

A shift to more positive potentials of an anodic polarographic wave indicates that the oxidation at the electrode is more difficult. In this particular investigation, the oxidation under study is that of the dropping mercury electrode to an oxidized form of mercury. This oxidation is facilitated by the removal of the oxidized form of mercury from the solution as a complex or an insoluble precipitate. In the case of the mitochondrial wave, the anodic process results in the formation of a mercury merceptide[5].

$$Hg + 2RSH \leftrightharpoons Hg(SR)_2 + 2H^+ + 2e \qquad (2)$$

If the mercaptan reacts with some substance which reduces the sulfhydryl concentration in the bulk of the solution, the anodic wave for the oxidation of the dropping mercury electrode will shift to more positive potentials. This is what was observed in the above experiments and these data indicate that both insulin and chromium(III) react with mitochondria, the site of reaction being the sulfhydryl groups of the mitochondrial membrane.

Two independent investigations suggest that the reaction between insulin and its receptor site goes via a sulfhydryl–disulfide linkage[13,14]. The above polarographic data support this observation. However, it should be remembered that polarographic investigations necessitate the use of nonphysiological concentrations of chromium and insulin. The observed effects of chromium(III) on the insulin–mitochondria reaction suggest that the role of chromium(III) is to facilitate the formation of the sulfhydryl–disulfide linkage by forming an intermediary ternary complex involving chromium(III), insulin, and the mitochondrial membrane.

ACKNOWLEDGEMENTS

The authors acknowledge the assistance of Mr. D. S. SKELLY in the preparation of mitochondria. The technical assistance of Mr. R. P. Taylor is gratefully acknowledged.

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