Communications

Kinetics of Electron Transfer between Azurin and Cytochrome 551 from *Pseudomonas*

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SUMMARY

The kinetics of electron transfer between the copper-containing protein azurin (Cu^{++}/Cu^{+}) and cytochrome 551 (Fe^{+++}/Fe^{++}) from *Pseudomonas* has been studied by rapid mixing methods. The reaction in both directions is fast; at low reagent concentrations ($\sim 10^{-6}$ M) the apparent second order rate constant, at 20°, is about 3×10^{6} M^{-1} sec^{-1} for the reaction $Fe^{++} + Cu^{++}$ and 1.4×10^{6} M^{-1} sec^{-1} for the reaction $Fe^{+++} + Cu^{+}$. At high reagent concentrations the rates tend to reach a limiting value indicating that the reaction is not a simple second order process.

The kinetics of the reactions of the reduced and oxidized forms of azurin and *Pseudomonas* cytochrome 551 with ferricyanide and dithionite has also been investigated. The rates of these reactions, at comparable reagent concentrations, are orders of magnitude lower than that between azurin and cytochrome 551.

Direct electron transfer processes between proteins containing oxidation-reduction groups are important from many points of view. These processes may have not only a relevant role in oxidative pathways in biological organisms, but also a special interest in connection with interpretation of mechanisms of electron transfer in general. The problems of electron transfer and electron exchange have been thoroughly investigated, both experimentally and theoretically in simple systems (1). It would be interesting to see how far this knowledge can be applied to proteins capable of undergoing an oxidation-reduction process at a specific site. For studies of this nature the choice of a suitable experimental model is critical. An ideal system appeared to be provided by the couples composed of the reduced and oxidized forms of the copper-containing protein azurin and of the heme-containing cytochrome 551 from *Pseudomonas*. Both proteins contain a single specific oxidation-reduction site per molecule; their oxidation-reduction reactions involve 1 electron; the reduced forms are stable in the presence of atmospheric oxygen; they can be purified easily; and many of their structural properties are known (2, 3). The present communication reports initial results on the kinetics of electron transfer between azurin and *Pseudomonas* cytochrome 551 which show that the very high rate of the process is associated with a striking degree of biological specificity.

Azurin was purified from *Pseudomonas fluorescens* as previously described (4). Cytochrome 551 was isolated in pure form from the final chromatographic step of the azurin purification procedure. Both proteins were essentially homogeneous in gel electrophoresis and in the ultracentrifuge. The sedimentation coefficient at protein concentration of 3 to 5 mg per ml was 1.2 S for cytochrome 551 and 1.63 S for azurin.

The copper content of azurin corresponded to 1 copper atom per 16,000 mol wt; the heme content of cytochrome 551 corresponded to 1 heme group per 9,000 mol wt. Following purification the proteins were obtained in the oxidized form. The reduced forms were prepared by treatment with a slight excess of dithionite followed by exhaustive dialysis against EDTA-containing buffer (0.1 M phosphate, pH 7, + 10^{-5} M EDTA). The same buffer was used in most of the experiments.

Spectrophotometric titrations of the reduced forms with ferricyanide gave end points corresponding to the expected stoichiometry of 1 mole of ferricyanide reduced per atom of copper or mole of heme oxidized. Oxidation or reduction of azurin was followed at 620 nm, and of cytochrome 551 at 550 or 416 nm. Kinetic experiments were performed with a Gibson-Durrum apparatus; the measured dead time was about 4 msec.

On mixing azurin and cytochrome 551 in different oxidation-reduction states, a rapid reaction takes place. The time course of the optical density changes at different wave lengths indicated that reduction or oxidation of copper occurred simultaneously with oxidation or reduction of an equivalent amount of heme. The dependence of the rate of reaction on reagent concentrations was studied at 416 nm under conditions in which cytochrome 551 concentration was constant and lower than that of azurin, which was varied over a large range. In both directions the time course of the reaction corresponded to a pseudo first order process (Figs. 1 and 2). However, the dependence of the rate constant on azurin concentration is complex. The shape of the curves represented in Figs. 3 and 4 indicates that the rate, at high azurin concentration, tends to reach a limiting value. The apparent second order rate constant corresponding to the initial slope of the curve is: 3×10^{6} M^{-1} sec^{-1} for the reaction $Fe^{++} + Cu^{++}$ and 1.4×10^{6} M^{-1} sec^{-1} for the reaction $Fe^{+++} + Cu^{+}$.

The total optical density change obtained in experiments of this type can be used to calculate the equilibrium constant for the over-all process. The values of K($[Cu^{+}]$ $[Fe^{+++}]/[Cu^{++}]$ $[Fe^{++}]$) corresponded to about 3.

The rate of the reaction in either direction depends little on pH between 4 and 9. The effect of temperature was studied in experiments similar to those of Figs. 3 and 4 between 15 and 35°; the apparent activation energy at low reagent concentrations (<10 μM) was about 17 kcal per mole in either direction.

To assess a significance to the results reported above, the following series of additional experiments was carried out.

1. Oxidized azurin was allowed to react with reduced mammalian cytochrome *c* under similar conditions to those used for the reaction with cytochrome 551. The reaction was slow, the apparent second order rate constant being about 10^{3} M^{-1} sec^{-1}, thus 10^{3} lower than that with bacterial cytochrome.

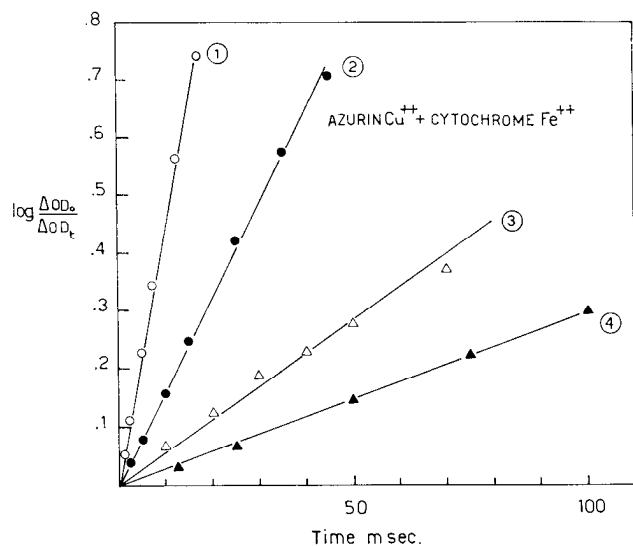2. The kinetics of the reaction of the reduced forms of azurin

FIG. 1. Time course of the reaction between oxidized azurin and reduced cytochrome 551. Potassium phosphate buffer, 0.1 M, pH 7 + 10^{-5} M EDTA. Wavelength, 416 nm. Temperature, 20°. Cytochrome concentrations, 5 × 10^{-7} M. Azurin concentrations: *1*, 5 × 10^{-5} M; *2*, 1.25 × 10^{-5} M; *3*, 3.13 × 10^{-6} M; *4*, 1.57 × 10^{-6} M. Zero time corresponds to about 4 msec after mixing.
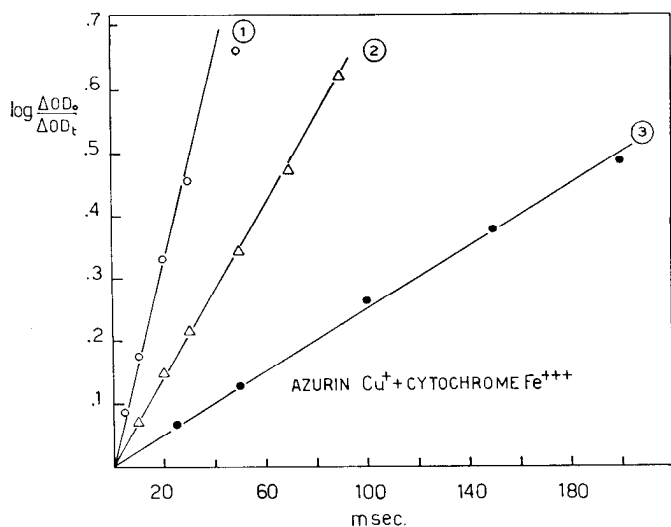

FIG. 2. Time course of the reaction between reduced azurin and oxidized cytochrome 551. Experimental conditions as in Fig. 1. Azurin concentrations: *1*, 5 × 10^{-5} M; *2*, 1.25 × 10^{-5} M; *3*, 3.13 × 10^{-6} M. Zero time corresponds to 4 msec after mixing.

and cytochrome 551 with excess ferricyanide was studied. The reaction appeared to correspond to a bimolecular process in both cases. The second order rate constant was 1.2 × 10^4 M^{-1} sec^{-1} for the reaction of azurin with ferricyanide and 8 × 10^4 M^{-1} sec^{-1} for the reaction of cytochrome 551 at pH 7 and 20°.

3. Experiments similar to the latter ones were performed on the reaction of the oxidized forms of azurin and cytochrome 551 with dithionite. In this case the rate depended linearly on dithionite concentration only at low concentrations. Under these conditions the apparent second order rate constant was about 4 × 10^3 M^{-1} sec^{-1} for azurin and about 1.5 × 10^4 M^{-1} sec^{-1} for cytochrome 551. At high dithionite concentrations, for both systems, the apparent second order rate constant tended to decrease as the dithionite concentration was increased.

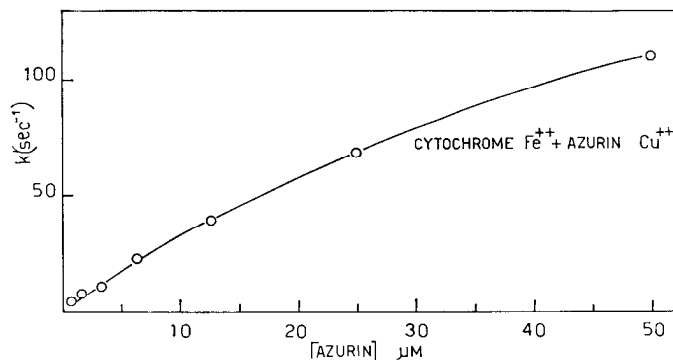These experiments show that electron transfer between azurin

FIG. 3. Pseudo first order rate constant as a function of azurin concentration in the reaction between oxidized azurin and reduced cytochrome 551. Cytochrome 551 concentration was 5 × 10^{-7} M in 0.1 M potassium phosphate buffer, pH 7, 10^{-5} M EDTA, 20°.
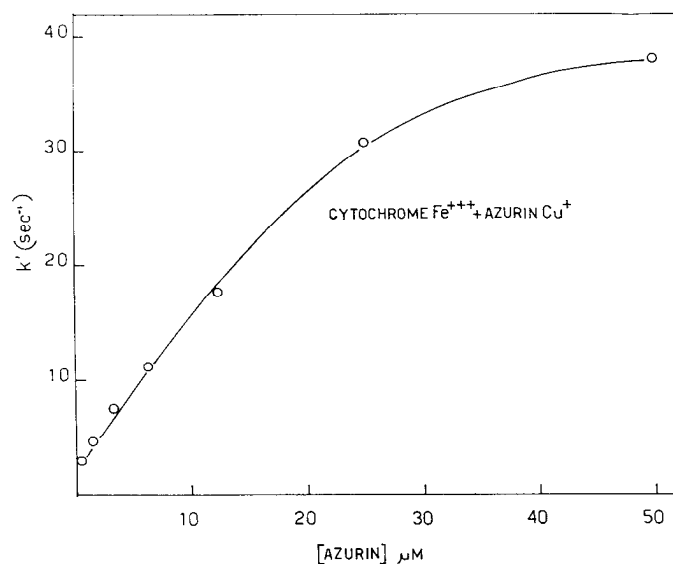

FIG. 4. Pseudo first order rate constant as a function of azurin concentration in the reaction between reduced azurin and oxidized cytochrome 551. Cytochrome 551 concentration was 5 × 10^{-7} M in 0.1 M potassium phosphate buffer, pH 7, 10^{-5} M EDTA, 20°.

and cytochrome 551 is a fast process involving a direct reaction between the two proteins. The reaction rate constant at low reagent concentrations approaches the higher values recorded in the literature for electron transfer reactions. The kinetic efficiency of the process is more striking if compared with the results on the reaction of the reduced and oxidized forms of the two proteins with ferricyanide and dithionite; the values of the second order rate constants in the latter cases are lower by one to three orders of magnitude than those corresponding to the direct reaction between azurin and cytochrome 551. At a higher level, the specificity of the electron transfer is indicated by the inadequacy of mammalian cytochrome *c* to substitute for the bacterial one, in spite of the similarity of the oxidation-reduction sites in the two proteins.

A detailed interpretation of the kinetics of the reaction between azurin and cytochrome 551 involves two types of problems, one related to the identification of individual steps within a reaction scheme, and the other connected to the microscopic mechanism of electron transfer.

The oxidation-reduction reaction between azurin and cytochrome 551 is complex and cannot be treated as a simple second

order process; in either direction, at high reagent concentration, the rate tends to approach a limiting value, indicating that under these conditions a monomolecular process may become rate-limiting. The data might be consistent with a general reaction scheme of the type

$$Cu^{++} + Fe^{++} \rightleftharpoons (C_1) \rightleftharpoons (C_n) \rightleftharpoons Cu^+ + Fe^{+++}$$
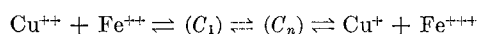
suggesting the rapid formation of one or more complexes (C) between the two proteins within which electron transfer takes place. Attempts to detect directly the presence of a thermodynamically stable complex between the various forms of the two proteins are presently being carried out.

An explanation of the mechanism of electron transfer between azurin and cytochrome 551, in analogy with lines developed for simple systems (1), would require knowledge of rates of electron exchange between oxidized and reduced forms of each protein. Such rates are not available at present, but experiments are being devised to estimate them.

A more detailed analysis of the kinetics of the system, hopefully including this additional independent information, will be presented in a forthcoming paper.

REFERENCES

1. REYNOLDS, W. L., AND LUMRY, R., *Mechanisms of electron transfer*, The Ronald Press Company, New York, 1966.
2. HORIO, T., HIGASHI, T., SASAGAWA, A., KUSAI, K., NAKAI, M., AND OKUNUKI, K., *Biochem. J.*, **77**, 194 (1960).
3. AMBLER, R. P., *Biochem. J.*, **89**, 341 (1963).
4. FINAZZI-AGRÒ, A., ROTILIO, G., AVIGLIANO, L., GUERRIERI, P., BOFFI, V., AND MONDOVÌ, B., *Biochemistry*, **9**, 2009 (1970).

Activation of Adipose Tissue Lipase by Skeletal Muscle Cyclic Adenosine 3',5'-Monophosphate-stimulated Protein Kinase*

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SUMMARY

A purified rabbit skeletal muscle adenosine 3',5'-monophosphate (cyclic AMP)-stimulated protein kinase enhanced lipolytic activity in adipose tissue homogenates. This effect required the presence of cyclic AMP and ATP and was completely blocked by a protein inhibitor of cyclic AMP-stimulated protein kinases. It is inferred that this effect is due to the phosphorylation and activation of a lipase in a system analogous to that involved in the activation of muscle glycogen phosphorylase.

It is well documented that cyclic AMP[1] mediates the lipolytic effects of many hormones in adipose tissue (1, 2); however, evidence for the mechanism of action of this nucleotide in stimulat-

ing the lipase activity has been lacking. Recently, an advance was made in the elucidation of that mechanism with the discovery of a cyclic AMP-stimulated protein kinase in adipose tissue and isolated fat cells (3). In muscle, the epinephrine stimulation of glycogenolysis is mediated by a cyclic AMP-stimulated protein kinase which initiates a series of protein phosphorylation reactions resulting in the conversion of glycogen phosphorylase to its active form (4–8). It was postulated that the adipose tissue protein kinase mediates the effects of cyclic AMP on a lipase or other enzymes (or both) in this tissue in a manner analogous to the role of the kinase in muscle (3). In this communication evidence is presented to support this hypothesis.

Although the most direct way to study the role of the adipose tissue protein kinase in lipolysis would be to investigate its ability to catalyze the phosphorylation and activation of a lipase from fat cells, this approach proved to be difficult because the purified kinase itself was found to be strongly contaminated with lipase. Efforts to remove this contaminant were not immediately successful because of the relatively small amount of starting material available when fractionating fat pad proteins. An opportunity to circumvent this difficulty was suggested by the finding that the cyclic AMP-stimulated protein kinases in general appear to present comparatively little specificity in regard to function and to tissue of origin. For example, it was found that the same protein kinase which is capable of catalyzing the activation of phosphorylase kinase also catalyzes the conversion of glycogen synthetase I to glycogen synthetase D (9). The lack of tissue specificity is illustrated by the experiment of Fig. 1. In this experiment the adipose tissue cyclic AMP-stimulated protein kinase was used to catalyze the activation of skeletal muscle phosphorylase kinase and was compared with the ability of the skeletal muscle protein kinase[2] to catalyze the same reaction. Identical amounts of the protein kinases from the two tissues, based on their ability to catalyze the cyclic AMP-stimulated phosphorylation of histone,[3] were used. The experiment shows that with two different amounts of each of the protein kinases the rates of phosphorylase kinase activation were almost as great with the adipose tissue enzyme as with the muscle enzyme. Further support for the idea that cyclic AMP-stimulated protein kinases from fat cells or from muscle are essentially identical is derived from a comparison of various kinetic parameters such as nucleotide specificity, pH optima, K_m values for cyclic AMP, and inhibition by a protein inhibitor purified from skeletal muscle (3, 7, 11, 12). These findings all served to suggest that, pending further purification of the adipose tissue protein kinase, the highly purified and readily available skeletal muscle enzyme could be used to study the mechanism by which cyclic AMP stimulates the lipase in adipose tissue.[4]

Sprague-Dawley rats (200 to 300 g) were anesthetized with intraperitoneal injections of sodium pentobarbital and the epididymal fat pads were removed and placed into tared flasks containing distilled water. After weighing the flasks the pads were

[1] The abbreviations used are: cyclic AMP, cyclic adenosine 3',5'-monophosphate; FFA, free fatty acids.

[2] The rabbit skeletal muscle cyclic AMP-stimulated protein kinase was prepared by a modification (10) of the procedure described by Walsh, Perkins, and Krebs (8). In this procedure the enzyme is separated into two peaks by column chromatography. The first peak to be eluted was used in the present study.

[3] A histone unit of protein kinase is that amount which catalyzes the transfer of 1 $\mu\mu$mole of ^{32}P to histone from γ-^{32}P-ATP in 1 min in an assay previously described (3).

[4] After completion of this study it was learned through a personal communication that Dr. Daniel Steinberg has also employed the skeletal muscle cyclic AMP-stimulated protein kinase in studying lipase activation.