Biochimica et Biophysica Acta, 637 (1981) 383–386
Elsevier/North-Holland Biomedical Press

BBA Report

BBA 41347

THE PARTICIPATION OF PRIMARY AMINO GROUPS OF SUCCINATE DEHYDROGENASE IN THE FORMATION OF SUCCINATE-Q REDUCTASE

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(Received March 16th, 1981)

Key words: Succinate dehydrogenase; Succinate-Q reductase; Protein modification; Fluorescamine; Ubiquinone

(1) Purified succinate dehydrogenase contains about 49 mol of lysine residues per mol enzyme. Titration of succinate dehydrogenase with fluorescamine indicates that half the lysyl groups are located on the surface of the protein and the other half are buried inside. (2) The reconstitutive activity and the low K_m ferricyanide reductase activity of succinate dehydrogenase decreased as the extent of alkylation of amino groups by fluorescamine increased. (3) The inhibitory effects of fluorescamine on both activities are parallel and are succinate concentration dependent. (4) Alkylation of the native succinate-Q reductase by fluorescamine does not affect the enzymatic activity or alter the enzyme kinetic parameters. This indicates that the inhibitory effect of fluorescamine on succinate dehydrogenase is due to the modification of a specific amino group(s) on succinate dehydrogenase which is essential in the interaction with QPs to form succinate–Q reductase. The participation of an ionic group in the formation of succinate-Q reducate supports the idea of the involvement of ionic interaction between succinate dehydrogenase and QPs.

Succinate dehydrogenase is a peripheral enzyme in the mitochondrial inner membrane. It is only loosely attached to the matrix side of the membrane. The methods employed in solubilization of this enzyme, such as the use of butanol in alkaline conditions [1] and chaotropic reagents [2], have led many investigators to stress the importance of hydrophobic interactions [2–5] between succinate dehydrogenase and the next adjacent components of the respiratory chain and membrane and to slight the involvement of ionic interaction. In fact, the release of succinate dehydrogenase from the respiratory chain by butanol under alkaline conditions is due to the alkaline treatment rather than butanol. Butanol merely denatures and precipitates the unwanted components [6]. The recent finding of low K_m ferricyanide reductase activity [7] in reconstitutively active succinate dehydrogenase and the direct correlation between this low K_m ferricyanide reductase activity and reconstitutive activity [8] imply that the 'site' exposed on succinate dehydrogenase resulting from the removal of the next component, now generally known to be a small molecular weight protein (QPs) [9–12], is at least partially ionic in nature [13].

Our recent studies on the interaction between pure succinate dehydrogenase and purified QPs have further substantiated the involvement of an ionic interaction between these two protein entities. It was found that the interaction between QPs and succinate dehydrogenase was ionic strength and buffer system dependent, and was especially sensitive to the anionic species of the buffer system [13]. The Cl^- of Tris-HCl buffer has a profound effect on the reconstitu-

Abbreviation: QPs, ubiquinone-binding protein in succinate-Q reductase.

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tion of succinate-Q reductase from soluble succinate dehydrogenase and isolated QPs. This indicates that some cationic group(s) in one or both of the proteins may be involved in the interaction. The cationic group most likely to be involved is a primary amino group. The alkylation of QPs with an amino group-specific reagent, fluorescamine, however, showed no significant effect on the reconstitutive activity of QPs [13]. This led us to investigate further the possible involvement of the amino group(s) of succinate dehydrogenase in reconstitution. In this communication we wish to report the effect of the alkylation of succinate dehydrogenase by fluorescamine on the reconstitution between QPs and succinate dehydrogenase, and the nature of the interaction between these two proteins.

The reconstitutively active, pure succinate dehydrogenase [6], and QPs [9] were prepared and assayed by the methods described previously. The reconstitution of soluble succinate dehydrogenase and QPs to form succinate-Q reductase [13,14] and the low K_m ferricyanide reducatse activity assay [14] were carried out by the reported methods.

Primary amino group content in succinate dehydrogenase was determined by fluorescamine. After reaction with fluorescamine, the sample was excited at 390 nm and the emission fluorescence was followed at 475 nm according to the method of Weigle et al. [15]. Fluorescence measurements were carried out on a Perkin-Elmer MPF-32 fluorescence spectrophotometer, which was made available to us by Professor Henry Tedeschi of the Department of Biology.

Fluorescamine was obtained from Sigma and prepared in anhydrous acetone. Other chemicals were obtained commercially at the highest available purity.

Amino acid analysis of pure succinate dehydrogenase [16] showed that 1 g of succinate dehydrogenase contains 487 μmol of lysine residues. When succinate dehydrogenase was subjected to fluorescamine titration (see Fig. 1) about half of the amino groups reacted readily with fluorescamine, indicating that these amino groups are probably located on the surface of the molecule. The remaining amino groups are less readily available to fluorescamine, and are probably more or less buried inside the molecule. The gradual decrease in fluorescence of fluorescamine after the first half of the lysine residues was alkylated

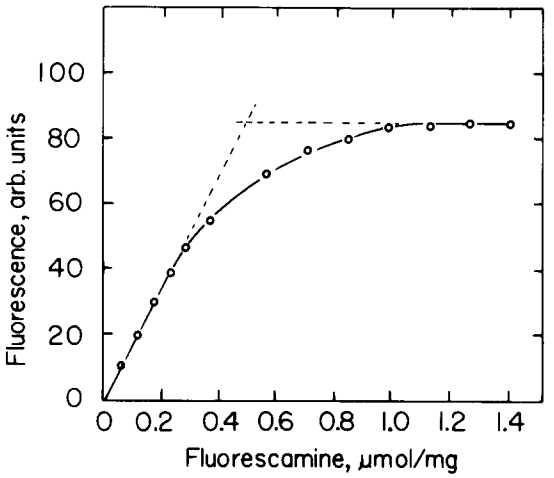

Fig. 1. Titration of primary amino groups in succinate dehydrogenase by fluorescamine. 3 ml of succinate dehydrogenase (0.31 mg/ml in 50 mM phosphate buffer, pH 7.4) were titrated with the indicated amounts of fluorescamine (26 mM in anhydrous acetone) at room temperature. The sample was excited in 390 nm and the emission fluorescence was followed at 475 nm.

could be explained by a slower reaction rate of the lysine residues with fluorescamine, thus increasing the hydrolysis of reagent in water to products which have no fluorescence [15]; or by the fact that the lysine residues are more buried and their fluorescence may be quenched by undetermined groups, possibly flavin or iron clusters. The latter explanation is less likely because the extrapolation of fluorescence yield in Fig. 1 gave a total of titratable lysine residues identical to that obtained by amino acid analysis [16].

The reaction of fluorescamine with amino groups occurs readily at room temperature but its solubility at 0°C is very low. Purified succinate dehydrogenase, however, is not stable at room temperature, and it is experimentally impossible to titrate the primary amino groups in succinate dehydrogenase with fluorescamine at 0°C under argon before reconstitution with QPs. An alternative experimental design was taken. A limited amount of succinate dehydrogenase and a given amount of fluorescamine were introduced simultaneously into succinate-Q reductase assay mixture and the solution was then mixed with excess QPs. The enzymatic activity of succinate-Q reductase was followed immediately by reduction of 2,6-dichlorophenolindophenol (DCIP). The concentration of succinate in the assay mixture was kept at 0.2 mM, much

lower than that of the regular assay condition, because the inhibition by fluorescamine was masked by a high concentration of succinate. The effect of fluorescamine on reconstitution is illustrated in Fig. 2. The alkylation of amino group(s) in QPs with fluorescamine has been shown to have no effect on its ability to reconstitute with succinate dehydrogenase to form succinate-Q reductase [13]. The effect of fluorescamine on reconstitution as shown in Fig. 2, must be attributed to the modification of succinate dehydrogenase. To substantiate this deduction, the effect of fluorescamine on the low K_m ferricyanide reductase activity of succinate dehydrogenase, which has been shown to be correlated with the reconstitutive activity of succinate dehydrogenase, was tested. Succinate dehydrogenase and fluorescamine were introduced to the assay mixture simultaneously. As expected, the results are identical to those of the reconstitution study (see Fig. 2). It should be mentioned that the concentration of fluorescamine used in Fig. 2 is relatively high as compared to that used in Fig. 1. The lower efficiency in inhibition is probably due to the low concentration of enzyme used under the experimental conditions. Under these conditions, part of the reagent used may have been hydrolyzed by water.

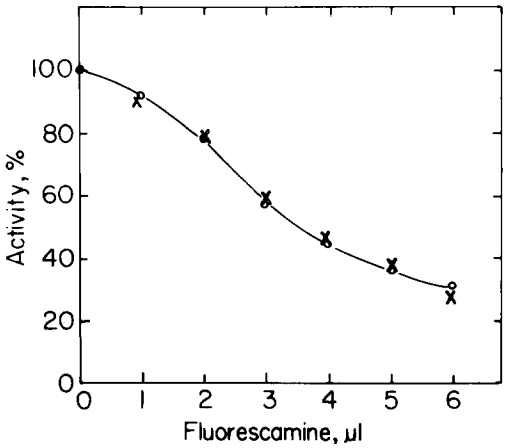

Fig. 2. Inhibition of low K_m ferricyanide reductase or reconstitutive activities of succinate dehydrogenase by fluorescamine. The assay mixture for low K_m ferricyanide reductase activity (o———o) contained 180 μM ferricyanide 0.2 mM succinate, 0.1% bovine serum albumin and 50 mM phosphate buffer, pH 7.4. The reduction of ferricyanide was followed at 420 nm immediately after the addition of 2 μl of succinate dehydrogenase (8.5 mg/ml and indicated amounts of fluorescamine. 1–6 μl of the fluorescamine solution (10.5 mM in acetone) were used. The reconstitutive activity (X———X) was determined by adding 5 μg of succinate dehydrogenase and the indicated amount of fluorescamine solution simultaneously into the succinate-Q reductase assay mixture containing 50 mM phosphate buffer, pH 7.4, 0.01% Triton X-100, 10 μM EDTA 30 μM Q_2, 50 μM DCIP, and 0.2 mM succinate, immediately followed by the addition of excess QPs (5 μg).

Fig. 3 shows the protective effect of succinate on succinate dehydrogenase. The inhibition of fluorescamine on succinate dehydrogenase is succinate concentration dependent. When the concentration of succinate in the assay mixture was above 2 mM, less than 30% inhibition was observed. However, at low concentrations of succinate, the inhibition was inversely proportional to succinate concentration. Data for succinate concentrations below 0.1 mM are difficult to obtain because succinate is used as substrate in this assay system. Quantitative comparison of the alkylation of succinate dehydrogenase in the presence and absence of succinate has not been made because the change in the amount of amino group(s) alkylated is rather small. This was expected, since only a small amount of amino groups participates in the interaction. The specific amino group(s) is believed to be located in the vicinity of the low K_m ferricyanide reductase active site (low K_m ferricyanide-binding site) and its accessibility to fluorescamine is very dependent on protein conformation.

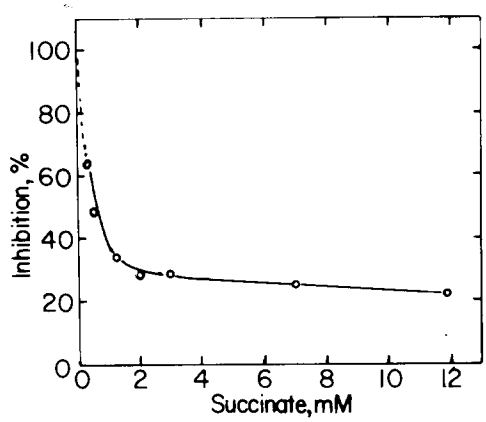

Fig. 3. Protective effect of succinate on the inactivation of low K_m ferricyanide reductase activity by fluorescamine. The experimental conditions were the same as those in Fig. 2, except that a constant amount of fluorescamine (5 μl) and various amounts of succinate were used. The activity was expressed in percent relative to that obtained in the absence of inhibitor at the corresponding succinate concentration.

When sufficient succinate is present, these specific amino groups are not accessible to fluorescamine treatment and very little inhibitory effect was observed. An alternative possibility is that these amino groups may be protected through the binding of succinate. The fact that the isolated succinate-Q reductase (complex II) and the reconstituted succinate-Q reductase were not sensitive to treatment with fluorescamine also indicates that the majority of amino groups on the surface of the enzyme are different from the specific amino group involved in interaction with succinate dehydrogenase. These results also indicated that the specific amino group(s) participating in interaction is exposed only when QPs is removed, and this particular amino group(s) in the intact system is not accessible to fluorescamine. The involvement of an ionic group(s) in the reconstitution of succinate-Q reductase from soluble succinate dehydrogenase and QPs advocates the role of ionic interaction between membrane-associated proteins.

Since the inhibitory effect of fluorescamine on the reconstitutive activity of purified succinate dehydrogenase is succinate dependent, and the concentration of succinate used in the assay mixture, as described in the previous paragraph, is slightly lower than the reported $K_m^{succ.}$ (0.56 mM) for the native enzyme, the possibility exists that the inhibition observed is due to the increase in $K_m^{succ.}$ of reconstituted succinate-Q reductase in the presence of fluorescamine rather than a true effect on reconstitution. To address this plausible argument, we have compared the $K_m^{succ.}$ of native and fluorescamine-treated succinate-Q reductase. Succinate-Q reductase (5 mg/ml) was warmed to room temperature and reacted with 0.05 vol. of an acetone solution of fluorescamine (20 mM) for 3 min and the mixture was returned to 0°C. The control experiment was done under the same conditions except that only acetone was used. The activity of succinate-Q reductase was measured at various concentrations of succinate, ranging from 0.1 to 10 mM. $K_m^{succ.}$ was determined from a double-reciprocal plot. No significant difference in $K_m^{succ.}$ for native and fluorescamine-treated succinate-Q reductase was found. $K_m^{succ.}$ was identical to the reported value of 0.56 mM. This result indicates clearly that the inhibition of fluorescamine on succinate dehydrogenase does indeed result from a decrease in efficiency of the reconstitution between succinate dehydrogenase and QPs and not from a change in the enzyme kinetic parameters of the reconstituted enzyme system.

We express our gratitude to Joy Steidl and Paul Haley for their technical assistance and to Professor Henry Tedeschi of the Department of Biology for the use of his fluorescence spectrophotometer. This work was supported by research grants from the National Institutes of Health (GM 26292) and National Science Foundation (PCM78 01394).

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