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ONE-ELECTRON TRANSFER REACTIONS IN BIOCHEMICAL SYSTEMS

VI. CHANGES IN ELECTRON TRANSFER MECHANISM OF LIPOAMIDE DEHYDROGENASE BY MODIFICATION OF SULFHYDRYL GROUPS

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SUMMARY

In the presence of NADH, lipoamide dehydrogenase (NADH:lipoamide oxidoreductase, EC 1.6.4.3) catalyzes reduction of *p*-benzoquinone by way of a mixed mechanism involving one- and two-electron transfers. The ratio of rates of semiquinone formation and NADH oxidation is 0.3, the two-electron transfer mechanism being dominant. When the enzyme is treated with cupric ions, phenylmercuric acetate, *N*-ethylmaleimide and NADH + arsenite the electron transfer mechanism is shifted to a one-electron transfer type accompanied with remarkable changes in the pH–activity curve of diaphorase activity. Upon treatment of the enzyme with Cu^{2+} a typical alkaline shift of pH optima for NADH–ferricyanide and NADH–benzoquinone reductase activities is observed. Changes taking place in the early phase of copper treatment can be mostly reversed by treatment with cysteine. It appears that the type of electron transfer mechanism is closely related to the extent of modification of lipoamide dehydrogenase by sulfhydryl reagents.

INTRODUCTION

The physiological role of lipoamide dehydrogenase (NADH:lipoamide oxidoreductase, EC 1.6.4.3.) is the catalysis of the reaction,

$$\text{Reduced } \alpha\text{-lipoic acid} + NAD^+ \rightleftharpoons \text{oxidized } \alpha\text{-lipoic acid} + NADH + H^+$$

It has been shown by Massey[1,2] and Searls and Sanadi[3] that this enzyme is identical with Straub's diaphorase which is known to catalyze an oxidation of NADH at the expense of artificial electron acceptors such as 2,6-dichloroindophenol (DCIP) and ferricyanide[4]. It has been also reported by Searls *et al.*[5], Casola *et al.*[6,7] and Ide *et al*[8]. that the loss of the physiological activity by chemical modifications of protein sulfhydryl groups is accompanied by a large increase in the NADH–DCIP (diaphorase) activity.

On the other hand, a series of experiments[9–11] have indicated that when quinones and oxygen are reduced by flavoprotein catalysis it is of importance to

Abbreviation: DCIP, 2,6-dichloroindophenol.

know whether the reduction occurs by way of a one-electron or two-electron transfer. Flavoproteins in electron transport systems catalyze a typical one-electron reduction of quinones while DT-diaphorase (NAD(P)H:(acceptor) oxidoreductase, EC 1.6.99.2) catalyzes a typical two-electron one[11]. In the case of xanthine oxidase (xanthine:O_2 oxidoreductase, EC 1.2.3.2), one-electron and two-electron reductions of *p*-benzoquinone or oxygen occur both at the same time, the ratio being dependent on the acceptor concentration[10]. This study is undertaken to determine the effect of sulfhydryl modification of lipoamide dehydrogenase upon the electron transfer mechanism of the diaphorase reaction.

MATERIALS AND METHODS

NADH and cytochrome *c* were obtained from Boehringer, Mannheim; DCIP from Merck; bovine serum albumin from Sigma Chemical Co. $CuSO_4$ and phenylmercuric acetate were purified by recrystallization from commercial supplies.

Lipoamide dehydrogenase was prepared by a method of Massey[1] from pig heart muscle. The concentration of the purified enzyme was calculated from the absorbance at 455 nm, with the use of ε_{mM} of 11.3. The copper-treated enzyme was prepared according to Casola *et al.*[6] by treatment with 8 gatoms of cupric per mole of enzyme flavin in 0.03 M potassium phosphate (pH 7.6) at 0 °C.

All enzyme assays were carried out at 25 °C in the presence of 0.67 mg bovine serum albumin per ml of reaction solutions. Buffers used were sodium acetate below pH 5.5, potassium phosphate between pH values 6.0 and 7.5, and Tris acetate above pH 8.0.

Absorbance changes during assays were recorded with the use of a Hitachi Model 124 spectrophotometer. Semiquinone concentration at steady state of reactions was measured with a Varian V-4500 x-band instrument, utilizing 100 kcycles field modulation and equipped with a flow apparatus.

RESULTS

Casola *et al.* have performed extensive studies on the sulfhydryl modification of lipoamide dehydrogenase with Cu^{2+} [6] or phenylmercuric acetate[7]. Using DCIP as an electron acceptor they reported an about 15-fold increase of the diaphorase activity when the enzyme was treated with Cu^{2+}. In their papers, however, the NADH–DCIP reductase activity only at pH 7.6 is described. Fig. 1 shows pH dependence of the DCIP activity of native and sulfhydryl-modified lipoamide dehydrogenases. As reported by Casola *et al.*[6,7] the increase in the activity depended on the amounts of Cu^{2+} and mercurial compounds and on the time of exposure to them. So, in this experiment the modification was carried out under the best condition so as to obtain a maximum diaphorase activity. At neutral pH a large increase of the diaphorase activity by sulfhydryl modification was observed, but at acidic pH the native enzyme itself exhibited a considerably high diaphorase activity and the extent of activation by the treatment with Cu^{2+} was rather small. The diaphorase activity of this enzyme has been measured using DCIP or ferricyanide as an electron acceptor. It was suggested[9–11] that the use of *p*-benzoquinone is profitable for the analysis of electron transfer reactions between the flavoprotein and electron acceptor. When

p-benzoquinone was used as an acceptor pH dependence of the diaphorase activity of lipoamide dehydrogenase became quite different from the NADH–DCIP activity, as indicated in Fig. 2. A maximal activity was observed at pH 5.8. In this case a

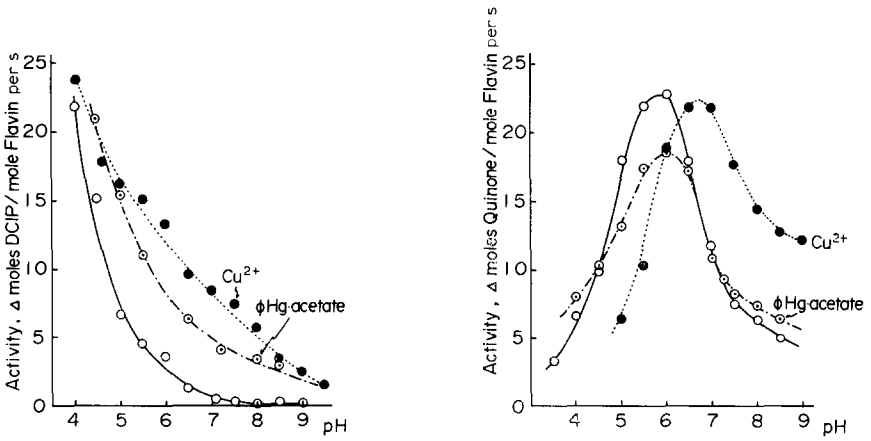

Fig. 1. Effect of pH on NADH–DCIP activity of native and modified lipoamide dehydrogenases. DCIP activity was measured spectrophotometrically at 600 nm. DCIP concentration was calculated from the absorbance at 600 nm, with the use of ε_{mM} of 21 at pH 7.0. Enzymes are: native (○), phenylmercuric acetate-treated (⊙; see Table II) and copper-treated (●; described in Materials and Methods). Concentrations: 0.1 μM enzymes, 100 μM NADH, 40 μM DCIP and 0.08 M buffers.

Fig. 2. Effect of pH on NADH–*p*-benzoquinone activity of native and modified lipoamide dehydrogenases. NADH–*p*-benzoquinone activity was measured spectrophotometrically at 340 nm. Enzymes used are the same as in Fig. 1. Concentrations: 0.06 μM enzymes, 100 μM NADH, 46 μM *p*-benzoquinone and 0.04 M buffers.

distinct shift of pH optimum of the NADH–*p*-benzoquinone reductase activity was caused by the treatment with Cu^{2+} without any essential change in the diaphorase activity at each optimal pH. The results also indicated that there is a great difference in pH–activity curves between two artifactual enzymes formed by copper-induced oxidation of sulfhydryl groups and by reaction with phenylmercuric acetate. At neutral pH, *p*-benzoquinone was a faster electron acceptor than DCIP for both native and copper-treated enzymes. The difference in the diaphorase activities for DCIP and *p*-benzoquinone was also observed in the time course of their activity change after Cu^{2+} was added to the lipoamide dehydrogenase solutions. The NADH–*p*-benzoquinone reductase activity reached a steady value much faster as compared with the case of DCIP (Fig. 3).

It has been pointed out[11,12] that in order to measure the ratio of one-electron and two-electron reductions of *p*-benzoquinone in the enzymic catalysis the use of cytochrome *c* as a scavenger of intermediate semiquinone is the most accurate method unless cytochrome *c* receives an electron directly from the enzyme. Fig. 4 shows *p*-benzoquinone-mediated reductions of cytochrome *c* during NADH–*p*-benzoquinone reductase reactions catalyzed by native and copper-treated enzymes. The native lipoamide dehydrogenase catalyzed a *p*-benzoquinone-mediated reduction of cytochrome *c* but the reduction was accelerated markedly by treatment of the enzyme with cupric ions. The involvement of one-electron reduction of *p*-benzoquinone was

thus clearly indicated in both reactions. A parameter, κ has been introduced in order to analyze the electron transfer mechanism quantitatively[12,13]. The parameter has been defined as a ratio of the rate of cytochrome c reduction to the rate of overall

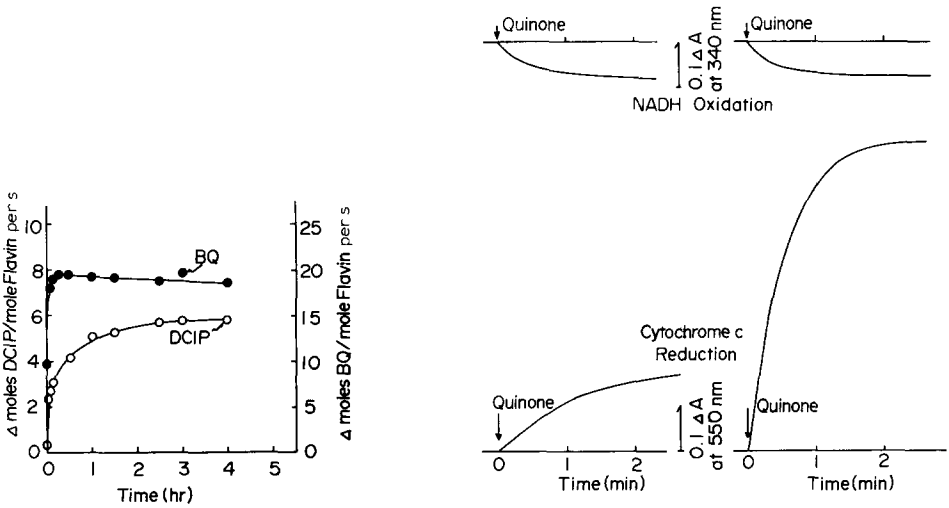

Fig. 3. Time course of changes in activity of NADH–DCIP and NADH–*p*-benzoquinone reductase reactions after Cu^{2+} was added. Lipoamide dehydrogenase (8 μM) was incubated with 64 μM Cu^{2+} in 0.03 M potassium phosphate (pH 7.6) at 0 °C. Aliquots were withdrawn at desired times and the reaction was stopped by addition of excess EDTA. Diaphorase activity was measured as follows: 0.053 μM enzyme, 100 μM NADH, 40 μM DCIP (○) or 50 μM *p*-benzoquinone (BQ, ●), and 0.04 M potassium phosphate (pH 7.2).

Fig. 4. *p*-Benzoquinone-mediated reduction of cytochrome *c* by native and copper-treated lipoamide dehydrogenases. Reactions were started by addition of *p*-benzoquinone. NADH oxidation was measured in the absence of cytochrome *c*. Upper and bottom diagrams indicate NADH oxidation and cytochrome *c* reduction, respectively. Concentrations: 0.1 μM native (left) or copper-treated (right) enzyme, 100 μM NADH, 18 μM *p*-benzoquinone, 40 μM cytochrome *c* and 0.05 M potassium phosphate (pH 7.2).

reaction (NADH oxidation in this case) for a particular reaction system[11,12]. The values of κ were calculated from the data in Fig. 4 to be 0.3 for the native enzyme and 1.94 for the copper-treated enzyme. The latter value indicates that the reduction of *p*-benzoquinone proceeds by way of a typical one-electron transfer. On the other hand, the value of 0.3 implies that the mechanism is a mixed type, the two-electron transfer mechanism being predominant. The results could be confirmed directly by the use of electron spin resonance (ESR) technique, which are shown in Fig. 5. Assuming that semiquinones formed as a consequence of one-electron transfer reaction decay only by disproportionation with rate constant, k_d, the value of κ can be estimated from the following equation,

$$\kappa = \frac{2k_d(SQ)_s^2}{v}$$

where v is the rate of overall reaction at steady state and $(SQ)_s$ is a steady state concentration of the semiquinone. The calculated ratio of κ between native and copper-

treated enzymes was 0.27:2 and was in good agreement with the result obtained by the scavenger method with the use of cytochrome *c* as a final electron acceptor. It was reported[10] that when *p*-benzoquinone is reduced by xanthine oxidase systems the

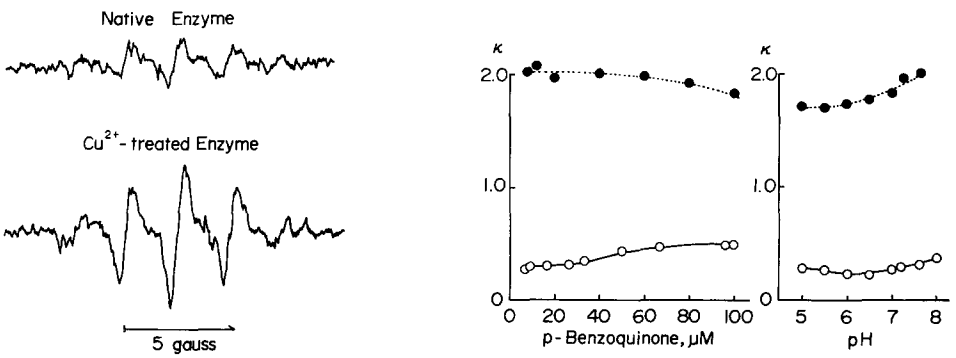

Fig. 5. ESR spectra of *p*-benzosemiquinone formed in the steady state of NADH–*p*-benzoquinone reductase reaction by native and copper-treated lipoamide dehydrogenases. Spectra were taken during continuous flow (1.2 ml/sec). The *p*-benzoquinone solution was mixed with the solution of enzyme and NADH. The time at the center of the cavity was about 0.1 s after the solutions were mixed. The rate of NADH oxidation was measured in different cells under the same conditions as ESR experiments and was estimated at 12 μM/s for the native enzyme and at 16 μM/s for the copper-treated enzyme. Concentrations (final): 0.9 μM native or 0.6 μM copper-treated enzyme, 100 μM NADH, 40 μM *p*-benzoquinone, and 0.1 M potassium phosphate (pH 7.0).

Fig. 6. Dependence of κ value upon *p*-benzoquinone and hydrogen ion concentrations. The value of κ was measured as a ratio of the rate of cytochrome *c* reduction to that of NADH oxidation. Concentrations: 0.1 μM native (○) or copper-treated (●) lipoamide dehydrogenase, 100 μM NADH and 40 μM cytochrome *c*. Left: 0.05 M potassium phosphate (pH 7.2). Right: 18 μM *p*-benzoquinone and 0.05 M buffers.

value of κ depends greatly upon the concentration of *p*-benzoquinone. In the case of lipoamide dehydrogenase, however, changes in the concentration of *p*-benzoquinone or in the pH caused no essential effect upon the κ value in the NADH–*p*-benzoquinone reductase reaction catalyzed by a native or copper-treated enzyme. The results are shown in Fig. 6. Independence of the κ value upon the pH is of particular interest because the NADH–ferricyanide reductase activity of these enzymes which is a compulsory one-electron transfer from the enzyme to an acceptor was markedly affected by the pH (Fig. 7). The shift of optimal pH caused by the sulfhydryl modification with Cu^{2+} was 1.3 for ferricyanide reaction and was slightly greater than in the case of NADH–*p*-benzoquinone activity. It should be noted that the specific activity for ferricyanide was about 5 times faster than for *p*-benzoquinone at each optimal pH.

Effect of cysteine on the copper-treated lipoamide dehydrogenase was studied by Casola *et al.*[6]. From Table I it can be seen that the changes in the electron transfer mechanism brought about by the reaction with Cu^{2+} was reversed to a large extent by treatment with cysteine, being accompanied with a decrease in the NADH–DCIP activity. Correlation between the NADH–DCIP activity and the value of κ was also observed when the enzyme was treated with phenylmercuric acetate, *N*-ethylmaleimide and NADH + arsenite. The results are summarized in Table II.

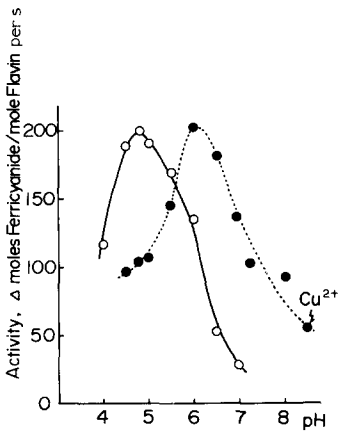

Fig. 7. Effect of pH on NADH–ferricyanide reductase activity of native and copper-treated lipoamide dehydrogenases. NADH–ferricyanide activity was measured spectrophotometrically at 240 nm, with the use of ε_{mM} of 1.05 for ferricyanide. Concentrations: 0.05 μM native (○) or copper-treated (●) enzyme, 200 μM NADH, 700 μM ferricyanide and 0.08 M buffers.

TABLE I

EFFECT OF CYSTEINE ON COPPER-TREATED LIPOAMIDE DEHYDROGENASE

Copper-treatment and cysteine dialysis were performed according to Casola *et al.*[6]. The enzyme was treated with Cu^{2+} as described in Materials and Methods and the reaction was stopped by addition of excess EDTA. The copper-treated enzyme was dialyzed against 0.03 M potassium phosphate (pH 7.6) containing 3 mM EDTA and 30 mM cysteine, and finally against the same solution without cysteine.

	DCIP activity *	*NADH–p-benzoquinone activity (moles/FAD per s)*	*Cytochrome c reduction (moles/FAD per s)*	κ **
Expt 1				
Native	1 650	6.9	2.40	0.35
Copper-treated (25 min)	18 700	15.0	26.0	1.73
Cysteine dialysis	3 320	9.9	5.56	0.56
Expt 2				
Native	1 880	7.4	2.25	0.30
Copper-treated (50 min)	21 000	14.0	25.0	1.78
Cysteine dialysis	9 150	11.7	9.7	0.83

* The unit of activity was defined by Savage[21] as a change in absorbance at 600 nm of 0.01 per min. DCIP activity used here was units per ml of enzyme divided by the absorbance of the enzyme at 455 nm[6].

** Ratio of the rate of cytochrome *c* reduction to that of NADH oxidation (see Fig. 4).

TABLE II

EFFECT OF VARIOUS SULFHYDRYL REAGENTS ON DCIP ACTIVITY AND κ VALUE

Treatment	*DCIP activity**	*κ value*
Native enzyme	1 500	0.31
Enzyme incubated with 2 equiv phenylmercuric acetate in 0.3 mM EDTA–0.03 M phosphate (pH 7.6) at c °C for 2 h	16 500	1.65
Enzyme incubated with 4 equiv *N*-ethylmaleimide in 0.1 M phosphate (pH 6.5) at 0 °C for 2 h	16 000	1.60
Enzyme incubated with 0.4 mM NADH + 1 mM arsenite in 0.03 M phosphate (pH 6.3) at 0 °C	21 000	1.75
Copper-treated enzyme prepared as described in Materials and Methods	25 000	2.0

* See first footnote of Table I.

DISCUSSION

It has been reported[5–7] that there is correlation between the loss of NADH–lipoamide reductase activity and the emergence of NADH–DCIP reductase activity when the enzyme is modified with sulfhydryl reagents. The treatment of lipoamide dehydrogenase with Cu^{2+} causes striking changes in its diaphorase activity. These changes can be measured in terms of the NADH–DCIP activity at neutral pH, but it is evident from the present results that the changes should not be called, in the strict sense, the emergence of diaphorase activity. The typical alkaline shift of pH-activity curves does occur when sulfhydryl groups of the enzyme are oxidized in the presence of Cu^{2+} though this may not be the case for other sulfhydryl modifications. A characteristic and common change caused by the modification of sulfhydryl and functional disulfide groups is a shift of electron transfer mechanism. This change can be measured in terms of κ value. From the data shown in Tables I and II, it would be concluded that for all modified enzymes there is rough correlation between the value of κ and the rate of NADH–DCIP reductase reaction measured at neutral pH.

A number of mechanisms have been proposed for lipoamide dehydrogenase reaction[5,7,14–18]. These reaction schemes have been presented as working hypotheses to explain spectral and activity changes caused by the modification of disulfide and sulfhydryl groups of the enzyme. The hypotheses have some common features which can be very roughly summarized as shown in Fig. 8. An intermediate involving a

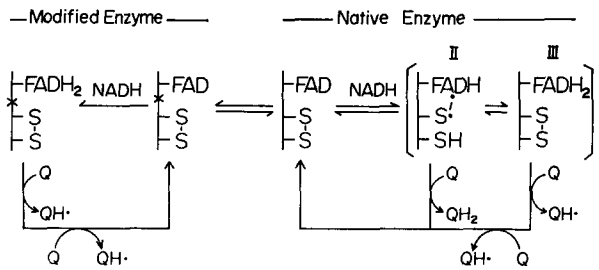

Fig. 8. Tentative scheme which shows the role of a functional disulfide group in the diaphorase reaction of lipoamide dehydrogenase. Intermediate II has been supposed to be a charge-transfer complex or a dimer of free radicals. Cross signs imply that interaction between FAD and disulfide group is lost.

specific interaction between the dithiol and flavin is considered to be responsible for the absorption peak at 530–535 nm in a reduced form of the native enzyme. Since the kinetic study by Massey *et al.*[22] it has been confirmed by many workers that this form is a catalytically active intermediate in the physiological reaction. Casola and Massey[7] have reported that this spectral species disappears upon the modification not only of a functional disulfide group but also of sulfhydryl groups. Experimental evidence in support of the correlation between sulfhydryl and functional disulfide groups has not yet been obtained[7].

Searls and Sanadi[3] suggested that NADH reduces the enzyme to form Intermediates II and III which are in equilibrium (Fig. 8), the former being responsible for the physiological reaction and the latter for the diaphorase reaction. The present results will suggest two types of the mechanism for diaphorase reaction of the native enzyme. Based on the scheme proposed by Searls and Sanadi[3], it might be concluded that Intermediate II reduces *p*-benzoquinone by the two-electron mechanism while Intermediate III reduces *p*-benzoquinone by the one-electron mechanism. This hypothesis seems very attractive but the possibility cannot be neglected that Intermediate II is the sole intermediate for the physiological and diaphorase reactions. In this case Intermediate II will transfer electrons to *p*-benzoquinone by a mixed mechanism in which $\kappa = 0.3$. Contrary to the hypothesis of Searls and Sanadi, it would appear that Intermediate II participates also in the diaphorase activity. For the phenylmercuric acetate-treated enzyme Casola and Massey[7] have shown that the fully reduced form of flavin may be an intermediate that reacts with DCIP. According to Veeger and Massey[23] in the case of the copper-treated enzyme, however, the formation of fully reduced flavin by NADH appears to be too slow to be involved in the diaphorase mechanism. The detail analysis needs further kinetic experiments which are now in progress in this laboratory.

The typical one-electron transfer mechanism has been found to be involved in the diaphorase reactions catalyzed by flavoproteins present in electron transport particles[11,12]. The only flavoprotein dehydrogenase that is known to catalyze a typical two-electron transfer from the enzyme to *p*-benzoquinone is DT-diaphorase. It was suggested[12] that this might be most characteristic feature of the DT-diaphorase in comparison with many other flavoprotein dehydrogenases which catalyze the reduction of quinones. Though the factors which differentiate such electron-transfer mechanisms are still hypothetical the present study will represent the following possibilities. The participation of sulfide in the reduced form of the flavin might facilitate two-electron transfer to *p*-benzoquinone by lowering the redox potential of the enzyme or by destabilizing a one-equivalent reduced form of the enzyme. Any changes in polarity which occur at the reaction site between flavin and acceptor might affect the mode of electron transfer reactions. A slight spectral shift of the flavin upon the treatment with Cu^{2+} reported by Veeger *et al.*[19,23] and Casola *et al.*[6] might indicate that the modified conformations have their FAD bound in a more polar environment[20].

Dramatic shifts of the pH–activity curve by treatment with Cu^{2+} might suggest an involvement of ionizable groups in the diaphorase activity. However, it seems unlikely that there is direct correlation between changes in the pK of the ionizable group and the mechanism of electron transfer from the enzyme to *p*-benzoquinone.

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