ON THE REACTION OF SUCCINATE DEHYDROGENASE WITH WURSTER'S BLUE AND ITS IMPLICATIONS ON THE EFFECT OF THE MEMBRANE ENVIRONMENT ON CATALYTIC ACTIVITY

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1. Introduction

It is well known that the turnover number of succinate dehydrogenase, as measured in the PMS–DCIP assay, declines substantially on extraction of the enzyme from the inner membrane. Thus, anaerobic extraction of the enzyme from ETP in the presence of succinate by the butanol procedure lowers the turnover number at 38°C from 21 000 ± 1000 to between 11 500 and 14 500, accompanied by a major decrease in the apparent K_m for PMS [1,2]. On reinserting the reconstitutively active, purified enzyme into the membrane the original high turnover number and K_m for PMS are restored. These observations have been interpreted to suggest a positive modulation of the enzyme, even in the activated state, by the membrane or one of its components, such as Q_{10} [1,2]. A similar conclusion was reached by Ernster's group [3] on the basis of different evidence.

An alternative explanation of these observations, advanced many years ago [4] is that PMS has two reaction sites near the dehydrogenase, one of which is lost on extraction from the membrane. Since both were thought to be needed to keep up with the rate of reduction of the enzyme by succinate, solubilization was thought to lower the measured activity in the PMS assay. This idea was abandoned [1,2], however, when it was noted [5] that reoxidation of the enzyme by PMS, at least in Complex II preparations, is not rate-limiting, because the EPR-detectable components of Complex II were reoxidized by PMS well within the turnover time of the enzyme, despite the fact that the preparation is depleted in Q_{10} and has a low turnover number in the PMS assay (~ 10 000).

Vinogradov et al. [6] confirmed the observation that the reincorporation of the dehydrogenase into membranes increases its activity in the PMS–DCIP assay but interpreted this to suggest that in membrane preparations Q_{10} acts as a second reaction site of PMS and that in soluble preparations lacking Q_{10} reoxidation of the enzyme by PMS is rate-limiting, despite the evidence cited to the contrary. Their conclusion rests on comparison of the rates of succinate oxidation by PMS and by Wurster's Blue (TMPD·, the semiquindiimine radical of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine) by purified succinate dehydrogenase before and after incorporation into alkali treated Keilin-Hartree preparations. They reported that the activity of the soluble enzyme in the TMPD· assay is 2–2.5-times higher than in the PMS–DCIP assay and while the activity in the PMS–DCIP assay increased by 60% on combination with the membrane, as previously reported [2], activity in the TMPD· assay did not.

Since this report, if confirmed, would require reinterpretation of the reasons why the turnover number of the dehydrogenase is much higher in intact membranes than in the isolated state and some other conclusions based on the widely used PMS–DCIP assay, we decided to compare critically the relative

Abbreviations: DCIP 2,6-dichlorophenolindophenol, DTT dithiothreitol, PMS phenazine methosulfate, TMPD· free radical of *N,N,N',N'*-tetra-methyl-*p*-phenylenediamine

rates of oxidation of succinate in the PMS–DCIP and in the TMPD· assays in various inner membrane and purified preparations. The results are presented below.

2. Materials and methods

TMPD·-free radical was prepared as described [7] and was twice recrystallized from 90% ethanol. The molar absorbances were, in agreement with published data [8,9], ϵ_{560nm} = 11 500 and $\epsilon_{510\ nm}$ = 5200. The purity of TMPD· was checked by titration with ascorbic acid. It also gave coincidental reductive titration curves and absorption spectra with a sample of TMPD· kindly provided by Dr A. Vinogradov. Succinate dehydrogenase assays were performed at 38°C or 25°C, as indicated, in 20 mM Tris–sulfate, 100 μM EDTA, 20 mM succinate, pH 7.5, with the inclusion of 1 mM KCN and 1 μg/ml of antimycin A in the assay of inner membrane preparations. PMS–DCIP and 'low K_m' ferricyanide assays were as in previous work [10,11]. The reduction of TMPD· was monitored at 560 nm at 20–100 μM dye concentrations and at 510 nm at 60–300 μM concentrations and activity was calculated by extrapolation to V_{max} (TMPD·). Although in the double reciprocal plots illustrated the dye concentration was varied in a < 10-fold range, so as to simulate the conditions used by others [6], the experiments were repeated using an > 10-fold range of dye concentration with identical results. Assays were started by adding the fully activated enzyme. ETP, Keilin-Hartree preparations and Complex II were activated at ~ 10 mg protein/ml at 38°C for 6 min in 0.2 M sucrose, 50 mM Hepes buffer, pH 7.4, the presence of 20 mM succinate, 1 mM KCN and antimycin A at 1 μg/mg protein. All enzyme preparations were isolated as in previous work [1,2].

3. Results and discussion

Figure 1B compares the rates of succinate oxida-

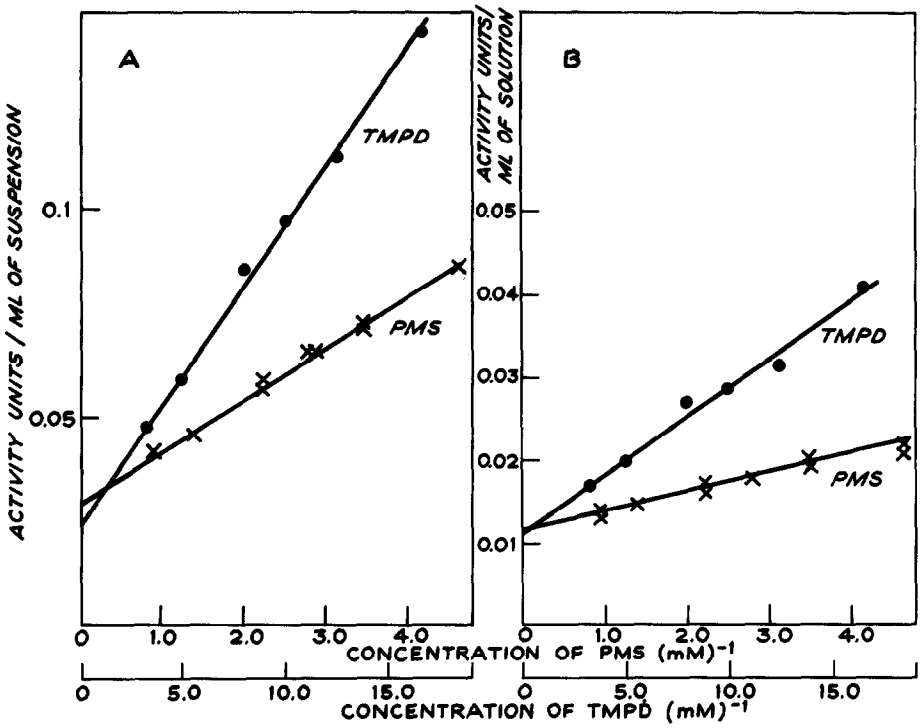

Fig.1. Comparison of the PMS and TMPD· reductase activities of (A) ETP and (B) of a reconstitutively active soluble enzyme extracted from ETP, at 38°C.

tion in a reconstitutively active, soluble preparation from ETP (turnover number = 12 500/min at 38°C) with PMS–DCIP and TMPD· as electron acceptors. As seen here, the two activities were identical. It has been shown [1,2] that the fraction of enzyme in such preparations which readily combines with the membrane and restores succinoxidase activity (usually 80–90% of the succinate dehydrogenase molecules) yields identical rates of succinate oxidation with PMS–DCIP and with low concentrations of ferricyanide ('low K_m' ferricyanide assay [11]) as electron acceptors. Hence, the reactivities of the reconstitutively active species with ferricyanide, PMS, and TMPD· seem to be equal under the experimental conditions. Figure 2 shows that on recombining this soluble preparation with alkali-treated ETP, the ratio of activities remains very near unity (cf. also table 1). Reconstitutively active soluble enzyme from Keilin-Hartree particles also gives equal activities with TMPD· and PMS–DCIP (fig.3A).

In the reconstitution experiment shown in fig.2 the turnover number of the succinate dehydrogenase in the reconstituted sample, following removal of the small fraction of uncombined dehydrogenase by centrifugation, was found to have increased from 12 500 in the soluble preparation to 18 000 in the PMS–DCIP assay and to 16 800 in the TMPD· assay. Thus, we cannot confirm the report [6] that on combination with the membrane reactivity with PMS increases but with TMPD· does not.

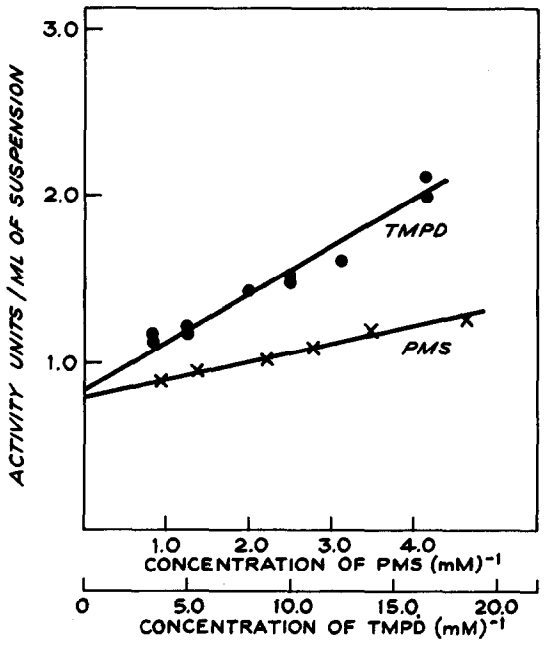

Fig.2. Comparison of the PMS and TMPD· reductase activities of the soluble enzyme used in fig.1B after reincorporation into an alkali-treated ETP preparation and reisolation of the reconstituted membrane sample. Assays at 38°C.

The only significant difference in the rates of succinate oxidation by these two electron acceptors we have noted was in the assay of inner membrane preparations, such as ETP (fig.1A) and Keilin-Hartree

Table 1
Reactivities of succinate dehydrogenase in the PMS–DCIP and TMPD· assays in various preparations

Preparation	Temp. (°C)	Specific activity[a]		Ratio	Turnover number
		PMS–DCIP	TMPD·	TMPD·/PMS	(PMS, 38°C)
Keilin-Hartree	38	0.92	1.05	1.14	
Soluble enzyme from Keilin-Hartree	38	29.8	29.8	1.0	9600
ETP	38	4.1	4.8	1.16	22 600
ETP	25	2.74	3.35	1.22	
Soluble enzyme from ETP	38	48.3	48.3	1.0	12 500
Soluble enzyme from ETP	25	29.0	32.8	1.13	
Reconstituted ETP	38	1.16	1.09	0.94	
Complex II	38	78.7	78.7	1.0	
SDB from Complex II	38	80.0	69.0	0.86	15 000

[a] μmoles succinate oxidized/min/mg protein at V_{max} with respect to dye

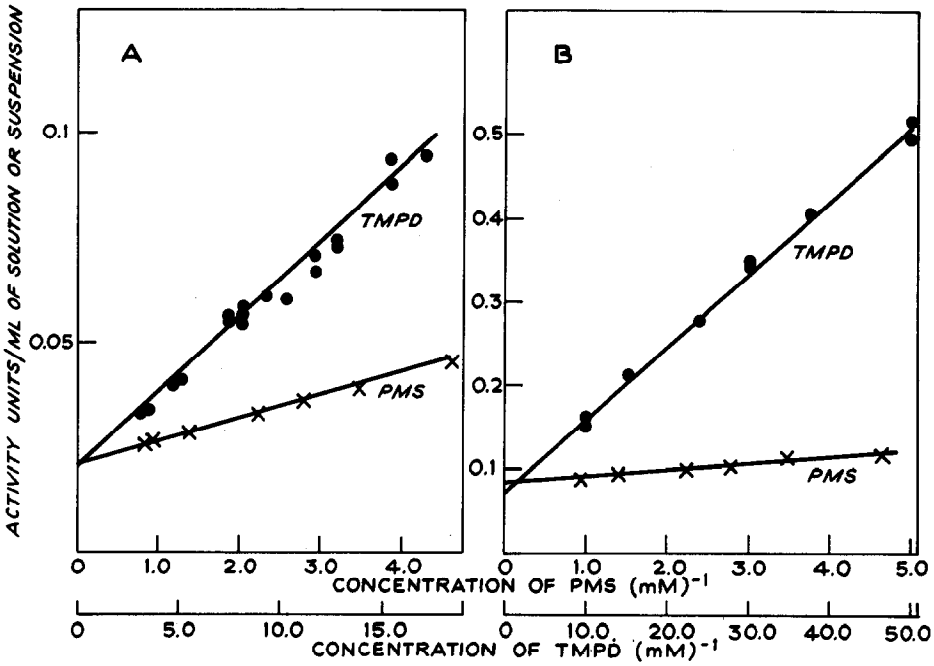

Fig.3. Comparison of the PMS and TMPD· reductase activities of (A) the soluble reconstitutively active enzyme extracted from a Keilin-Hartree preparation and (B) of a Keilin-Hartree preparation at 38°C.

particles (fig.3B). Even in these instances the difference amounted only to 14–16% (table 1). In several other experiments with ETP the turnover number was from 3–20% higher in the TMPD· than in the PMS–DCIP assay. While even this small difference has not been noted in the soluble samples and thus does not substantiate the claim that in purified preparations the rate of reoxidation of the enzyme by PMS is limiting, it is of interest, since both CoQ, and its saturated analog, 2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone, give the same turnover number for succinate dehydrogenase in antimycin-inhibited ETP as does PMS [12].

Table 1 summarizes the results on the reactivity of succinate dehydrogenase with PMS and TMPD·, respectively, in various enzyme preparations. For comparison we have included a Complex II preparation and a sample of soluble succinate dehydrogenase ('SDB') extracted from Complex II with 0.8 M perchlorate in the presence of succinate and DTT under argon, under the conditions of Davis and Hatefi [13]. Both preparations are devoid of significant Q_{10} and in neither was the activity with TMPD· higher than with PMS, although their turnover numbers are well below that of ETP [1]. The activity of the SDB sample in the 'low K_m' ferricyanide assay was only ~ 20% of the activity in the PMS–DCIP assay, which is of interest since no more than 17–22% of the dehydrogenase in the Davis-Hatefi preparation is reconstitutively active [14] and since there is substantial reason to believe that activity in the 'low K_m' ferricyanide assay and reconstitution activity go hand-in-hand [1,11].

The data hitherto discussed were obtained in assays at 38°C, the temperature chosen in order to permit comparison of the turnover numbers with the majority of data in the literature. Concerned about our inability to reproduce the findings of Vinogradov et al. [6], we looked for differences in technique. Since the only difference we found was that their assay had been conducted at ~ 25°C, we repeated some key experiments at this temperature. As seen in table 1, the ratio of the specific activities of ETP in the TMPD·/PMS–DCIP assays increased only slightly (from 1.16 to 1.22), while in the soluble preparation the ratio was ~ 14% higher at 25°C than at 38°C.

Although this is probably a bit beyond experimental error, it is far from the 2–2.5-fold greater activity reported for the soluble enzyme in the TMPD· assay [6].

An unambiguous test of our contention that reoxidation of the enzyme by PMS is not rate-limiting is that all components of the enzyme active in electron transport should be reoxidized by PMS at least as fast as predicted from the turnover number. We have previously reported that all EPR-detectable components of Complex II reduced by succinate are reoxidized completely in < 6 ms at 0°C on rapid mixing with succinate [5]. Dr H. Beinert has now kindly repeated this experiment with a reconstitutively active soluble enzyme from ETP (turnover number = 13 500 at 38°C) as used in fig.1B. On mixing the enzyme ($\sim$ 50 μM), previously reduced with 20 mM succinate, with an equal volume of 2 mM PMS at 16°C in the rapid-freeze apparatus and subsequent quantitation of the EPR signals, both the $g = 1.94$ (center 1) and the $g = 2.01$ (HiPIP) Fe–S centers, as well as the flavin radical were completely oxidized at 6 ms and reoxidation was virtually complete at $\sim$ 3 ms. This corresponds to a turnover number of at least 10 000–20 000 at 16°C while the catalytic reaction showed a turnover number of 4900 at this temperature. Hence, reoxidation of succinate dehydrogenase by PMS does not seem to be rate-limiting in soluble preparations and thus modulation of the catalytic activity by the membrane or by one of its components remains the best available explanation for the increased activity observed on combination with the membrane.

Acknowledgements

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