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MAGNETIC SUSCEPTIBILITY OF SUCCINATE DEHYDROGENASE: THE 4-IRON PREPARATION

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SUMMARY

Measurements were made at 20°C of the magnetic susceptibility of soluble succinate dehydrogenase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1) (4 Fe/flavin) from beef heart. The resting enzyme and enzyme in the presence of fumarate or oxaloacetate appeared to be nearly diamagnetic. The addition of succinate, malonate, or malonate *plus* succinate, and other treatments resulted in an increase in paramagnetism: $\Delta\chi_{M,Fe} = 1200 \cdot 10^{-6}$ c.g.s. units (average) per enzyme iron atom. An excess of dithionite caused a larger increase in paramagnetism: $\Delta\chi_{M,Fe} = 2500 \cdot 10^{-6}$ c.g.s. units (average). The change due to dithionite was partially reversible upon autoxidation. We cannot account quantitatively for these changes in terms of the paramagnetic species observable by electron spin resonance.

INTRODUCTION

We report initial observations on the magnetic susceptibility of succinate dehydrogenase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1), and the results are compared with parallel data from electron spin resonance (ESR) studies. While the data are insufficient to permit detailed discussion, we wish to point out that rather large increases in susceptibility are observed at 20°C upon reduction and that these increases cannot be accounted for by paramagnetic species detected by ESR either at room temperature or 77°K. In this regard the magnetic properties of succinate dehydrogenase show a similarity to those reported previously for xanthine oxidase[1,2].

EXPERIMENTAL METHODS

Enzyme

Soluble succinate dehydrogenase was prepared from beef heart after the method of SINGER *et al.*[3]. This preparation (4 Fe/flavin) has good stability at 20°C in air and was chosen for these initial experiments over other less stable preparations (6–8 Fe/flavin)[4,5]. The preparation was found to be 0.20 mM in succinate dehydrogenase flavin and 0.795 mM in non-heme iron and to contain 160 mg protein/ml. Estimated purity was about 25 %. Heme iron and acid extractable flavin were not detected. Contaminating proteins were assumed to be diamagnetic and the data seem consistent

with this assumption. Turnover numbers for the preparation (flavin basis) were 950 min^{-1} (unactivated) and 3480 min^{-1} (activated with succinate) at 22°C in the presence of 20 mM succinate and 2.5 mM phenazine methosulfate[6].

Magnetic susceptibility

Measurements were made at 20°C with the differential susceptometer of THEORELL AND EHRENBERG[7], equipped with a plexiglass sample tube of about 0.3-ml volume. The instrument was calibrated using acidic $NiCl_2$ solutions ($\chi_{M,20°C} = 4434 \cdot 10^{-6}$ c.g.s. units), which gave $2.0 \cdot 10^{-11}$ c.g.s. volume susceptibility units per instrumental unit.

Electron spin resonance

First derivative spectra of ESR absorption were obtained at 77°K from samples in quartz tubes (3-mm inner diameter), using a Varian X-band spectrometer. Second integrals of ESR signals were determined through a computer program based on data processed originally by a TMC CAT-1024. Non-saturating microwave power levels were used.

The standard for spin counts was 1 mM cupric–EDTA. The concentrations of ions and radicals giving ESR signals were estimated by correcting the second integrals for spin transition probability and population factor according to the approach of AASA *et al.*[9] and AASA AND VÄNNGÅRD[10]. The population factor was taken to be 1.0 for $S = 1/2$ systems (radicals at $g = 2.00$, non-heme iron at $g = 1.94$ and 2.01, and cupric–EDTA) and 0.33 for the signal at $g = 4.3$. This signal is considered to be an approximately isotropic line of high-spin ferric ion ($S = 5/2$) in a rhombic ligand field[9,11].

RESULTS

As shown in Fig. 1 the values can be grouped in three susceptibility levels. Level I is observed with the enzyme as prepared and seems to persist in the presence of fumarate or oxaloacetate. A control involving the addition of the enzyme's buffer indicated little or no change as the result of time or handling during an experiment. The average of the Level I values is 398 instrument units.

An average diamagnetic correction used for proteins is 2.5 instrument units·ml·mg^{-1}. While this correction is approximate and depends in detail on the composition of the protein, it suggests that 400 instrument units represent approximately a diamagnetic state for the enzyme preparation. The existence of an ESR signal at about $g = 4.3$[8] indicates, however, that the preparation must have some residual paramagnetism due to high-spin ferric iron[9]. Table I indicates that the paramagnetic contribution could be as much as -32 instrument units ($810 \cdot 10^{-6}$ c.g.s. units per enzyme iron atom), assuming $14800 \cdot 10^{-6}$ c.g.s. units for the molar susceptibility of these ferric ions.

The amplitude of the $g = 4.3$ signal was unaffected by malonate, fumarate, oxaloacetate, and succinate under the conditions of our experiments. It was abolished, however, by dithionite and reappeared completely after autoxidation of excess dithionite, as described by DER VARTANIAN *et al.*[8]. Several cycles of freezing and thawing of the enzyme preparation had little effect on the amplitude of the $g = 4.3$ signal.

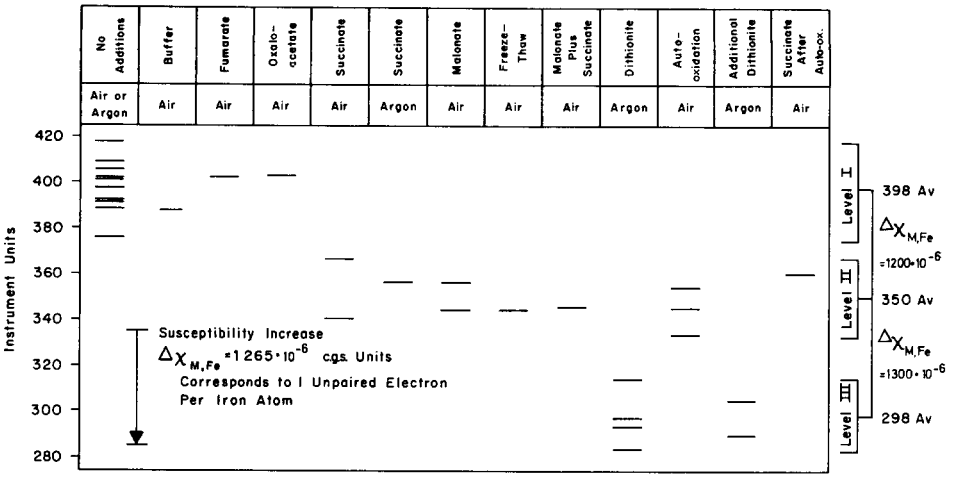

Fig. 1. Magnetic susceptibility of succinate dehydrogenase at 20° C. The lines indicate the values in terms of instrument units from separate experiments with succinate dehydrogenase (0.20 mM in flavin and 0.79 mM in iron) in 0.2 M Tris–HCl, 3 mM EDTA (pH 7.75). The headings indicate the ingredients added in an experiment and the gas phase. "Buffer" represents an experiment in which 5 μl of the enzyme's buffer was added to 500 μl of enzyme solution. The final concentrations of dicarboxylic acids were 4–8 mM, and those of dithionite were 3–5 mM. The reagents were added as 0.5 M stock solutions and caused dilutions of the enzyme of less than 2% total. The values have been corrected for the diamagnetic change resulting from this dilution. Each value has an error due to instrument effects of from ± 4 to about ± 10 instrument units. A solution 1 mM in a $S = 1/2$ species with molar magnetic susceptibility, χ_M, of $1265 \cdot 10^{-6}$ c.g.s. units would cause a deflection of -63 instrument units. Paramagnetism increases from top to bottom.

The paramagnetic increase from Level I to Level II of $1200 \cdot 10^{-6}$ c.g.s. units per enzyme iron atom can be realized by the addition of succinate, or malonate, or following the autoxidation of dithionite, or even after cycles of freezing and thawing of the enzyme preparation. Of these, only succinate causes the appearance of ESR signals in the $g = 2$ region due to radicals (component at $g = 2.00$) and due to reduction of non-heme iron complexes (asymmetric signal with components at $g = 1.94$ and $g = 2.01$)[8]. As computed in Table I, these $S = 1/2$ systems would appear to contribute up to $210 \cdot 10^{-6}$ c.g.s. units per iron atom. This contribution is small and corresponds to the addition of about 2/3 of an unpaired electron per flavin. Similarly, radical signals observed at room temperature[12–14] could account for changes of only $200 \cdot 10^{-6}$ c.g.s. units or less per iron atom.

Oxygen may be able to bind reversibly to soluble succinate dehydrogenase in the presence of succinate[12,15]. One might expect to observe a considerable difference (about $750 \cdot 10^{-6}$ c.g.s. units per iron atom) between systems with and without oxygen, if the bound oxygen is in the triplet state and at a concentration equivalent to the flavin concentration (0.2 mM). Large differences were not evident, and, in fact, the paramagnetism of Level II is not obviously related to oxygen. It is apparent, therefore, that oxygen if bound is diamagnetic.

Level III values are observed in the presence of dithionite under anaerobic conditions. We observe reversibility between Level III and Level II over one and probably two cycles of reduction. Again, it is not possible to account for the changes in susceptibility from the double integration of ESR signals. In the presence of excess

TABLE I

ESTIMATION OF HYPOTHETICAL CONTRIBUTIONS TO THE MAGNETIC SUSCEPTIBILITY OF SUCCINATE DEHYDROGENASE AT 20° C FROM PARAMAGNETIC SPECIES OBSERVABLE BY ESR AT 77°K

The samples ($200 \cdot 10^{-6}$ M flavin, $790 \cdot 10^{-6}$ M Fe), were prepared as indicated in the legend to Fig. 1, and the conditions and computations were as described under EXPERIMENTAL METHODS.

ESR species and g values	*Additions to enzymes*	*Double integral of ESR signals* *	*Estimated concentration of ESR species* ** ($M \cdot 10^6$)	*Stoichiometry* $\frac{[ESR\ species]}{[Fe]}$	*Assumed spin number*	*Assumed molar magnetic susceptibility of ESR species* ($\chi_M \cdot 10^6$ *c.g.s. units*)	*Hypothetical contribution of ESR species to magnetic susceptibility of enzyme iron*	
							Incremental instrument reading	$\Delta\chi_{M,Fe} \cdot 10^6$ *c.g.s. units*
High-spin ferric $g = 4.3$	None or succinate	40–60	43	0.054	5/2	14 800	−32	810
Reduced non-heme iron $g = 1.94, 2.01$ *plus* radicals $g = 2.00$	Succinate under argon	80–120	133	0.17	1/2	1260	− 8.4	210
Same as above	Dithionite under argon	110–150	167	0.21	1/2	1260	−10.5	260

* Relative to the double integral from 10^{-3} M cupric–EDTA, which is assigned a value of 1000.

** Based on maximum value of double integral corrected for spin transition probabilities and population factors. See EXPERIMENTAL METHODS.

dithionite, the non-heme iron and radical signals contribute up to $265 \cdot 10^{-6}$ c.g.s. units per iron atom, which is equivalent to about 4/5 of an unpaired electron per flavin. The net increase in susceptibility due to species observed by ESR would have to be less than this, because the paramagnetic contribution of the high-spin ferric ions would be expected to decrease in the presence of dithionite, which causes the $g = 4.3$ signal to disappear. The extent of the change is uncertain, since low-spin or high-spin ferrous ions could result upon reduction.

DISCUSSION

A lack of data on susceptibility as a function of temperature and the rather low enzyme purity prevented an accurate determination of the diamagnetic contribution to susceptibility. It is clear, however, that the enzyme as prepared (Level I) is only weakly paramagnetic at 20°C and it is likely that much of this paramagnetism can be accounted for by the high-spin ferric species observed by ESR at $g = 4.3$.

Level II may represent several non-identical magnetic states, since it can be reached by different treatments which affect the ESR spectrum differently. Changes in oxidation–reduction, spin state, or degree of magnetic coupling among iron atoms may be involved[16–19]. Also possibly relevant regarding Level II are activation of the enzyme upon exposure, for example, to malonate, and partial denaturation, as might occur upon refreezing and thawing. However, the freeze–thaw cycle cited in Fig. 1 had virtually no effect on activity or ESR spectrum.

The additional effect of dithionite (Level III) also cannot be explained unequivocally at this time. Dithionite is able to reduce succinate dehydrogenase preparations containing 7 to 8 iron atoms per flavin by as many as 8 electron equivalents[8]. The reductive titration of our preparations (4 Fe/flavin) by dithionite has been followed optically, and preliminary results suggest that reduction involves at least 4 and possibly 6 electron equivalents. The results are consistent with the idea that flavin reduction consumes 2 equivalents and a pair of iron atoms yielding the characteristic non-heme iron signal consumes a third equivalent. The present results suggest that additional equivalents would involve reduction of remaining iron complexes to give more strongly paramagnetic species not observed by ESR. The rather large increase in paramagnetism following reduction by dithionite is atypical of non-heme iron proteins which can accept only 1 or 2 electron equivalents, but resembles that observed for xanthine oxidase ($\Delta\chi_{M,Fe} = 2000 \cdot 10^{-6}$ c.g.s. units for dithionite)[1,2] which, like succinate dehydrogenase, can accept several electron equivalents per flavin[20,21]. It is not known whether the development of strongly paramagnetic states upon reduction is a general property of ferroflavoproteins.

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