An Analysis of some Thermodynamic Properties of Iron–Sulphur Centres in Site I of Mitochondria

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1. The midpoint potentials of the various iron–sulphur centres in Site I were determined at different pH values by the technique of redox potentiometry. An interesting feature is the pH-dependence of Centre N-2, the highest potential component of the NADH dehydrogenase segment of the respiratory chain. 2. The apparent midpoint potentials of Centre N-2 (NADH dehydrogenase) and S-1 (succinate dehydrogenase) and their pH-dependence was also determined by using the succinate/fumarate couple. Again Centre N-2 is pH-dependent in midpoint potential, and Centre S-1 is not. The results obtained by titrating with the succinate/fumarate couple are in quantitative agreement with those obtained for these centres by redox potentiometry. 3. Oxidation–reduction titrations of iron–sulphur centres with the couple NADH/NAD⁺ and an analogue APADH/APAD⁺ in the presence of rotenone gave results substantially different from those obtained by redox potentiometry; these differences may be due to the mechanism of action of NADH dehydrogenase and its specific interaction with NADH. 5. The addition of ATP to an NAD⁺/NADH-poised system induces an uncoupler-sensitive oxidation of Centre N-4.

Historically the mitochondrial respiratory chain has been divided into segments (sites, loops or complexes). The region of the respiratory chain mediating the transfer of reducing equivalents from NADH to ubiquinone is known as Site I in its energy coupling, and Complex I (NADH–ubiquinone reductase) (Hatefi *et al.*, 1962) in its protein chemistry. Complex I contains flavin and at least five distinct iron–sulphur centres (Orme-Johnson *et al.*, 1974; Ohnishi, 1975; Albracht *et al.*, 1977). Two of these iron–sulphur centres appear to be of the binuclear type (2Fe–2S) and three of the tetranuclear type (4Fe–4S) (Albracht & Subramanian, 1976; Ohnishi, 1979). The flux of

reducing equivalents through Site I in mitochondrial preparations can be coupled to the synthesis of ATP from ADP and P_i. It is probable that this coupling occurs through the intermediary of a proton electrochemical gradient ($\Delta\mu_{H^+}$), but whether this gradient is built up by a proton-translocating redox loop or pump is not known.

Some studies on the effect of $\Delta\mu_{H^+}$ on the distribution of reducing equivalents within Site I have been published: Gutman *et al.* (1972) have reported an ATP-induced oxidation of Centre N-2 (see the footnote and Scheme 1 for nomenclature) by NAD⁺ in the presence of piericidin A, an inhibitor of ubiquinone reduction by Site I. This represents an apparent lowering of the midpoint potential (E_m) of this component. However, by manipulating the conditions it is possible to shift the apparent midpoint potential of Centre N-2 to either higher or lower values on ATP addition (Ohnishi, 1976). Ohnishi (1976) has reported that (in the absence of a Site-I inhibitor) the midpoint potential of Centre N-2 can be raised, the donor being either the succinate/fumarate couple or a redox-dye system. This observation raises the question of the nature of the energy input between succinate dehydrogenase and Centre N-2, a region not hitherto considered to have a 'coupling site'.

Other ATP-induced effects on Site I are the finding of a higher midpoint potential for Centre N-1 (reductant NADH/NAD⁺) by Gutman *et al.* (1975), a

Abbreviations used: iron–sulphur centres associated with Complex I are denoted by N- followed by a number (N-1a, N-1b, N-2---N-5) and those associated with succinate dehydrogenase by S- (S-1 to S-3) (see Scheme 1); $\Delta\mu_{H^+}$, proton electrochemical gradient; E_h , electrode potential, either measured directly or calculated from the poise of the substrate couple; APAD(H), acetylpyridine–adenine dinucleotide (reduced); Mes, 4-morpholine-ethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid; Taps, 3-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]-1-propanesulphonic acid; *n*-value, the number of reducing equivalents transferred on oxidation or reduction of one equivalent of the centre in question.

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Scheme 1. Major iron-sulphur centres of the mitochondrial respiratory chain

The box on the left hand side represents NADH dehydrogenase, and contained within this box and denoted by N- are the iron-sulphur centres of this enzyme. The box on the right hand side represents succinate dehydrogenase, and contained within this box and denoted by S- are the iron-sulphur centres of this enzyme. The numbers represent E_m in mV.

result at variance with those reported in the present paper. Ohnishi (1976) divides the Centre N-1 content into two centres, N-1a and N-1b, and finds an apparent ATP-induced lowering of the midpoint potential of Centre N-1a in a redox-dye system. Consideration of this observation of Gutman *et al.* (1975) would exclude Centre N-1 from the hypothetical Site-I redox loop formulated by Mitchell (1972) and Garland *et al.* (1972). Ohnishi's (1976) finding is difficult to interpret owing to complications imposed by the differential permeability and electromobility of reduced and oxidized Methyl Viologen, the major redox dye under her experimental conditions (Jones *et al.*, 1976).

The reducibility of Centre N-1a by NADH has also been questioned and it is possible that, like Centre S-2 of succinate dehydrogenase, it is not in equilibrium with the rest of the respiratory chain.

In this paper, the pH-dependences of the midpoint potentials of iron-sulphur centres are reported. We also report an ATP-dependent oxidation of Centre N-4 in the NADH/NAD⁺-poised system and point out anomalies in the redox behaviour of Centres in Site I.

Methods

Pigeon heart mitochondria were prepared as described by Chance & Hagihara (1963). Ox heart mitochondria were prepared as described by Löw & Vallin (1963). Submitochondrial particles were prepared in the presence of MgATP as described by Beyer (1967), except that a French press was used for breakage.

The titrations with the substrate couple were performed anaerobically under an argon atmosphere in

the same vessel in which the redox titrations were performed; a separate incubation was performed for each E_h value.

Protein was measured by the biuret method (Gornall *et al.*, 1949).

In redox titrations, where the samples were to be studied by e.s.r., the sample was poised at ambient room temperature and then clamped by rapid freezing to 81 K and stored at 77 K until examination. Such treatment can lead to two types of artifact: the midpoint potential of a centre may be significantly temperature-dependent or the decrease in temperature may cause a significant change in pH. In practice, the former does not seem to present much of a problem: the midpoint potentials of several respiratory-chain components were determined by both low-temperature e.s.r. and room-temperature optical spectroscopy and found to be in close agreement, possibly because, on freezing, the centres can no longer react with the dye (or substrate) couple. In a multienzyme system, where redistribution of reducing equivalents may be possible at very low temperatures and where some components are pH-dependent, temperature-alteration-induced changes in pH could be a problem; pH changes induced by freezing have been investigated by Williams-Smith *et al.* (1977) and Orii & Morita (1977); both groups found that the smallest artifacts were obtained when zwitterionic buffers were used and relatively high concentrations of protein were present. In our experiments, high protein concentrations are an experimental requisite and we have not seen significant pH anomalies with Tris/HCl or phosphate buffer, although we have largely confined ourselves to zwitterionic buffers.

E.p.r. measurements were performed with a

Varian-E4 spectrometer. The temperature of the sample was controlled with a variable temperature cryostat (Air Products, model LTD-3-110). The temperature was measured with a carbon resistor installed just below the bottom of the e.p.r. sample tube. The field was calibrated by the use of crystalline diphenylpicrylhydrazyl free radical and a commercial weak-pitch standard.

Results

pH-dependence of the midpoint potentials of the iron-sulphur centres of the respiratory chain assayed by potentiometric redox titration

(i) *Centre S-1.* The midpoint value of this centre is not pH-dependent in the pH range 6.5–8.5 (Fig. 1a).

(ii) *Centres N-1a and N-1b.* Centre N-1b appears to have a pH-independent midpoint potential, whereas Centre N-1a exhibits a pH-dependent midpoint potential throughout the pH range tested (Fig. 1a).

(iii) *Centre S-3.* Midpoint potentials of Centre S-3 fluctuate considerably from preparation to preparation. Recent detailed analysis of overlapped e.p.r. signals arising from Centre S-3 and from a spin-coupled ubisemiquinone pair (Ingledew *et al.*, 1976) has revealed the existence of some modulation mechanism of the former midpoint potential by the latter pair (Ohnishi *et al.*, 1977). Higher Centre S-3 midpoint potentials are obtained where the structural integrity around the ubisemiquinone pair (which is believed to be an electron acceptor of Centre S-3) is more intact. A close comparison of midpoint values at different pH values within the same batch of mitochondria clearly shows the pH-independence of Centre S-3 (Fig. 1b).

(iv) *Centre N-2.* This has a pH-dependent midpoint potential, but its redox behaviour shows a peculiar anomaly. The centre appears to be able to exhibit one of two possible midpoint potentials. At pH 7.0 these are approx. -20 mV and -140 mV .

Fig. 1. *pH-dependence of midpoint potentials of various iron-sulphur centres*

The Figure is split into three sections to avoid congestion. (a) shows the relationship between midpoint potential and pH for Centres S-1, N-1b and N-1a. (b) shows the behaviour of Centre S-3 and the anomalous results obtained for Centre N-2. The behaviour of Centre N-3 is shown in (c). ○, ●, Ox heart mitochondria (representing two separate batches of material); □, pigeon heart mitochondria; ▲, ox heart submitochondrial particles; △, pigeon heart submitochondrial particles. In pigeon heart systems all titrations were performed on different batches. Potentiometric titrations were conducted in a medium containing 50 mM buffer (Mes, Mops, HEPES or TAPS depending on pH). E.p.r. conditions were selected for titration of different centres as described by Ohnishi (1976).

Within one batch of mitochondria the midpoint potential is consistently all one value. It is not clear why this happens but both forms of Centre N-2 are pH-dependent throughout the range tested (Fig. 1b).

(v) Centres N-3, N-4 and N-5. The midpoint potential of Centre N-3 is pH-independent (Fig. 1c). Similarly Centres N-4 and N-5 showed no pH-dependence in midpoint potential (results not shown).

Analysis of the apparent E_m values of the iron-sulphur centres when substrate couples are used to poise redox potentials (E_h)

(a) Titration of Centres S-1 and N-2 by using the succinate/fumarate couple at different pH values in submitochondrial particles. The midpoint potential of Centre N-2 was pH-dependent (as is the succinate/fumarate couple), whereas that of Centre S-1 was pH-independent (Fig. 2); both of these centres showed n -values of 1.

(b) Titration of N centres by using NADH/NAD⁺ and APADH/APAD⁺ couples under uncoupled conditions in the presence of rotenone. (i) Centre N-1. A value of $E_{m7} = -287$ mV ($n = 2$) was obtained for Centre N-1b. Centre N-1a was apparently not reduced by NADH (Fig. 3). An n -value of 2 gives a better fit to the data obtained than does the expected value of 1.

(ii) Centres N-3 and N-4. Both these centres titrate as apparent $n = 2$ components when titrated with NADH/NAD⁺. The trough in the Centre N-3 titration is very pronounced (Fig. 4), much more so than in the potentiometric redox titrations. The slight

Fig. 2. pH-dependence of midpoint potentials of Centres S-1 and N-2 measured by using succinate/fumarate couple. Pigeon heart submitochondrial particles (10 mg of protein/ml) in 20 mM buffer + 100 mM-KCl were used. Mes, Hepes and Tris/HCl were used for pH 6.5, 7.5 and 8.5 respectively. Carbonyl cyanide 3-chlorophenylhydrazone (17 μ M) and KCN (1.3 mM) were also present. The succinate/fumarate ratio was varied between 100:1 and 1:100, the total concentration of the couple being 25 mM.

Fig. 3. Redox titrations of Centre N-1b with the NADH/NAD⁺ and APADH/APAD⁺ couples

Experiments were conducted anaerobically in 50 mM-Mops, pH 7.0, containing 80 μ g of rotenone/ml and 5 μ M-carbonyl cyanide 4-trifluoromethoxyphenyl-hydrazine. Pigeon heart submitochondrial particles (12 mg/ml) were titrated by the appropriate couple at a total concentration (reduced plus oxidized) of 5 mM. Points obtained with the APADH/APAD⁺ couple are denoted by squares. Solid and open triangles denote different batches of submitochondrial particles.

Fig. 4. Redox titrations of Centres N-3 and N-4 with the NADH/NAD⁺ couple

Experimental conditions were the same as in Fig. 3. The arrows delineate the approximate midpoint potentials. $\circ$, Centre N-4 ($g = 1.88$); $\bullet$, Centre N-3 ($g = 1.86$). Excluding the troughs, the lines drawn follow a theoretical $n = 2$ curve.

dip in the titration of Centre N-4 is due to an overlap of this signal with that of Centre N-3. The midpoint potentials of Centres N-3 and N-4 measured in this way are approx. -295 mV (N-4) and -305 mV (N-3). These are quite different values from those obtained by potentiometric titration (both -245 mV at pH 7.2; Ohnishi, 1975). The trough in the N-3 titration has a minimum around -360 mV. A similar trough has been observed in potentiometric redox titrations performed on NADH dehydrogenase and ascribed to a dipole-dipole interaction between Centre N-3 and the flavin semiquinone (Salerno *et al.*, 1977).

(c) Redox titrations with NADH/NAD⁺ in the presence of added ATP. (i) Centres N-3 and N-1b

Fig. 5. Titrations of Centres N-1, N-3 and N-4 with the NADH/NAD^+ couple in the presence (a) and absence (b) of ATP. Experiments were conducted anaerobically in 50 mM-Mops, pH 7.0, containing 2 mM- MgCl_2 , 80 μg of rotenone/ml and pigeon heart submitochondrial particles (12 mg of protein/ml). (a) Results obtained in the presence of 5 μM -carbonyl cyanide 4-trifluoromethoxyphenylhydrazone; (b) results obtained in the presence of 10 mM-ATP. The top axis denotes $\log ([\text{NADH}]/[\text{NAD}^+])$, addition of NADH alone and addition of dithionite with Benzyl Viologen as mediator (DT+BV).

showed no significant ATP-dependent alteration in their apparent midpoint potential. The trough in the titration of Centre N-3 in the presence of ATP was either absent or much smaller under these conditions (Fig. 5).

(ii) Centre N-4 is apparently oxidized throughout the redox-potential range obtainable with NADH;

Fig. 6. E.p.r. spectra showing g_x of Centres N-3 and N-4 in the presence and absence of ATP

E.p.r. conditions: microwave frequency, 9.05 GHz; modulation amplitude, 1×10^{-3} T; microwave power, 10 mW; sample temperature, 9.5 K. Experimental conditions were as in Fig. 3, except where indicated. (a) +10 mM-ATP and 5 μM -carbonyl cyanide 4-trifluoromethoxyphenylhydrazone; (b) +10 mM-ATP. The $[\text{NADH}]/[\text{NAD}^+]$ ratio was 5:1 and the total concentration 5 mM.

this indicates a lowering of the apparent midpoint potential of Centre N-4 by at least 115 mV. This phenomenon is indicated more clearly in the e.p.r. spectra shown in Fig. 6.

(iii) Centre N-2. Under almost all conditions investigated in this paper Centre N-2 was reduced. Oxidation of Centre N-2 on ATP addition could occasionally be observed when APAD⁺, a higher-potential analogue of NAD⁺, was used as acceptor. Such an effect is predicted from the results of Gutman *et al.* (1972).

Discussion

Although transfer of reducing equivalents between iron-sulphur centres and cytochromes is usually thought of in terms of electron flow, protons are also involved in redox reactions. Such an involvement is inferred from the pH-dependence of the midpoint potential. A pH-dependence of about 59 mV/pH unit implies, from the Nernst relationship, a stoichiometry of one proton/electron bound. An involvement of protons in redox reactions has been reported for mitochondrial *b* cytochromes (Wilson *et al.*, 1972a), *a* cytochromes (Wilson *et al.*, 1972b) and the Rieske iron-sulphur centre (Prince *et al.*, 1975). The question as to whether or not protons are involved in these reactions in the time scale of electron flow has only been thoroughly investigated in one instance (Petty *et al.*, 1977), where it was demonstrated that the protons associated with the *b*-type cytochrome in *Rhodospseudomonas sphaeroides* do indeed turnover at the same rate as the electrons.

Table 1 summarizes our findings. Two components show pH-dependence in midpoint potential, Centre N-2 and Centre N-1a. Centre N-1a is the one component in Site 1 that does not appear to be reduced by NADH. Two centres apparently have a 'coupling site' between themselves and the site of NAD⁺/

NADH interaction with the respiratory chain; these are Centre N-4 (present work) and Centre N-2 (Gutman *et al.*, 1972). We have not been able to determine whether the apparent lowering of the midpoint potential is caused by the total $\Delta\mu_{H^+}$ or by its component forces individually.

In the titrations in which the succinate/fumarate couple was used and the redox states of Centres S-1 and N-2 were monitored the results obtained are similar to those obtained by redox potentiometry. However, a different result is obtained for the lower-potential NADH dehydrogenase centres when the results of substrate and potentiometric titration are compared.

There are two discrepancies: the centres of Site 1 do not titrate as $n = 1$ components but approximately $n = 2$, and the measured midpoint potentials are slightly lower when the substrate couple is used. These observations may be due to the fact that NADH is an obligatory two-equivalent reductant and is feeding these into a closed system (rotenone is present). It thus follows that the centres ($n = 1$) will be reduced in pairs with apparent n -values of 2. The centres of succinate dehydrogenase will, on the other hand, be able to equilibrate with centres in other succinate dehydrogenase molecules through the quinone pool, this latter being an open system.

If, in NADH dehydrogenase, pairs of electrons are being fed into a closed system, then, as NADH is a two-equivalent reductant and the iron-sulphur centres are one-equivalent oxidants, a transformer is required between the two; this function is probably carried out by the flavin moiety, which can accept two electrons simultaneously and transfer them sequentially. In such a system the iron-sulphur centres must exhibit an apparent n -value of 2 when titrated with NADH. However, if the system is not effectively closed and extensive inter-dehydrogenase electron transfer occurs, then an alternative explanation for the phenomenon must be sought.

Table 1. Apparent midpoint potentials (mV) and apparent n -values of mitochondrial iron-sulphur centres assayed under different conditions

The redox centres are as listed in Scheme 1. Abbreviation: ND, not determined.

Redox centre	Redox potentiometry			NAD ⁺ /NADH titration			Succinate/fumarate titration	
	E_{m7}	n -value	$\Delta E_m/\Delta pH$	E_{m7}	n -value	$E_{m7} + \text{ATP}$	E_{m7}	n -value
N-1a	-370	1	60	—	—	—	—	—
N-1b	-245	1	0	-287	2	-330	—	—
N-2	-20	1	60	—	—	—	-20	1
N-3	-245	1	0	-305	2	-315	—	—
N-4	-245	1	0	-295	2	-400	—	—
N-5	-270	1	0	ND	—	—	—	—
S-1	0	1	0	—	—	—	0	1
S-3	+100	1	0	—	—	—	—	—

The ATP-induced oxidation of Centre N-4 in a NADH/NAD⁺-clamped system is hitherto unreported. Gutman *et al.* (1972) have previously reported an oxidation of Centre N-2 by NAD⁺ in the presence of piericidin A on ATP addition. This represents reversed electron flow through a fairly large but undefined redox potential difference, with no other iron-sulphur centre either initially reduced or subsequent to ATP addition oxidized (except Centre N-2). However, as NAD⁺ reduction is a strictly $n = 2$ step, the oxidation of Centre N-2 by NAD⁺, in a closed system, requires a second electron from somewhere. What could this source be? Ubiquinol or ubisemiquinone is one possibility, as quinones are known to be associated with Complex 1 (King *et al.*, 1978).

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