Biochem. (Life Sci. Adv.) 1988, 7 : 71 - 75

Uncoupling of mitchondrial energy transfer by closely related uncouplers: Relevance for the question of localized coupling modes.

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ABSTRACT

The relationship between rate of energy-dependent transhydrogenation, membrane potential and mode of energization has been studied in submitochondrial particles under conditions where the membrane potential is the major component of the protonmotive force. Using structurally similar uncouplers, it has been shown that the rate of transhydrogenation correlates with the magnitude of the membrane potential in a manner independent from the source of energy, i.e., ATP hydrolysis or succinate oxidation. In contrast, with two of the four uncouplers used, the type of supporting reaction had a quantitative influence on both inhibition of transhydrogenation and attenuation of the membrane potential.

The effect of a pair of uncouplers on a number of energy-dependent mitochondrial reactions was also examined. It was found that the ratios of uncoupler concentrations necessary for half-maximal inhibition were neither constant nor a monotonous function of the uncoupler sensitivities of the reactions. These data are consistent with a limited contribution of local coupling modes to mitochondrial energy transduction.

INTRODUCTION

It is generally accepted that mitochondrial energy transduction proceeds through protonic coupling (Westerhoff et al., 1984). In the chemiosmotic theory, electrogenic proton pumps generate and utilize a delocalized electrochemical gradient composed of membrane potential and ΔpH (Mitchell, 1981). One of the corollaries of this concept is that the rates of energy-dependent processes should be determined solely by the magnitude of this gradient, and not by the means used to modify the latter.

In the last few years, data presented in a number of reports appeared to be at variance with this postulate (Rottenberg & Hashimoto, 1986, for earlier references see Kell & Westerhoff, 1985; Westerhoff et al. 1984). Most recently, Rottenberg & Hashimoto (1986) showed that changes in the P/O ratios effected by fatty acids, ionophores or classical uncouplers correlate with the proton electrochemical gradient in entirely different ways. These and other data have lead to proposals of localized proton stores and coupling schemes with more than one pathway of energy transfer (Rottenberg & Hashimoto, 1986, Westerhoff et al., 1984). In particular, the involvement of a proton capacitor closely associated with the ATP synthase has been postulated for mitochondria (Rottenberg & Hashimoto, 1986) and possibly detected in chloroplasts (Junge et al., 1984). This might be the molecular basis for earlier observations that, in some cases, uncoupling of energy-dependent transhydrogenation discriminates between ATP hydrolysis and succinate oxidation as sources of energy (Schuermann & Hanstein, 1980).

In the present paper, we study the relationship between mode of energization, membrane potential and rate of transhydrogenation as influenced by uncouplers. As a further test for delocalized coupling, the uncoupling characteristics of a set of energy-requiring reactions has been investigated with two closely related uncouplers. While indications for more than one type of energy transfer exist, our data show a relationship between transhydrogenation rates and protonmotive force which is independent of mode of energization.

MATERIALS AND METHODS

Rat liver mitochondria and beef heart submitochondrial particles were prepared by published procedures (Kaschnitz et al., 1976; Löw & Vallin, 1963). Reverse electron transport (Phelps & Hanstein, 1977), ATP-P_i exchange (Pullman, 1967), ATP hydrolysis (Pullman et al. 1960), ADP/O ratio, respiratory control, succinate and 3-hydroxybuty-

rate oxidase, protein concentration (Hanstein & Hatefi, 1974), octanol-water coefficients (Fujita et al. 1964), pK_a values (Hanstein et al., 1979), ACMA fluorescence quenching and potential-sensitive shifts of oxonol VI (Kiehl & Hanstein, 1984) were measured as described.

Energy-dependent transhydrogenation was measured at 30° essentially according to Lee et al. (1964) in a medium containing 200 mM sucrose, 50 mM Tris-sulfate pH 7.5, 10 mM $MgSO_4$, 0.5 mM NADH, 0.25 mM NADP, 1 mM oxidized glutathion, 0.5 U/ml glutathione reductase, submitochondrial particles (0.2-0.4 mg/ml), and 5 µg/ml rotenone. The energized reaction was started by the addition of 5 mM succinate or 2.5 mM ATP.

Membrane potential and pH-gradient in submitochondrial particles were determined from the distribution of [14-C]-thiocyanate and [14-C]-methylamine, respectively, across the vesicle membrane at room temperature essentially as described (Rottenberg, 1979). To 2.9 mg submitochondrial particles suspended in 495 µl buffer containing 200 mM sucrose, 10 mg $MgSO_4$, 50 mM Tris sulfate pH 7.5, 2.5 µg rotenone, 2.5 µCi [3-H]-sucrose, 1.25 µCi [14-C] -methylamine, 10 µM methylamine (or 1.25 µCi [14-C]-KSCN, 12.5 µM KSCN) and 1 µg catalase were added 5 µl succinate (1 M) containing 2% H_2O_2, or 5 µl Na-ATP (0.5 M). At the concentrations of succinate, oxygen and protein used, the membrane potential as measured by the oxonol response was constant for 8-9 minutes. After 2 minutes, duplicate aliquots of 175 µl each were sedimented in an airfuge (Beckman) at maximum speed (160,000 g). The supernatant was mixed with 50 µl sodium dodecylsulfate (10%) and 10 ml of a dioxane-based scintillation cocktail, allowed to stand overnight and analyzed in a two-channel scintillation counter. The pellet was dissolved in 50 µl sodium dodecylsulfate (10%) and, together with a 160 µl rinse, mixed with 10 ml of the counting cocktail and counted as above. After correction of the 14-C/3-H spill-over and the contribution of the interstitial volume (4.7 ± 0.2 µl/mg protein), determined from the [14-C]-saccharose distribution in pellet and supernatant, $\Delta\psi$ and ΔpH were calculated from the concentrations of SCN- and CH_3NH_2 inside and outside, using an internal volume of 0.75 µl/mg protein (Sorgato et al., 1982). Oxonol VI (Kiehl & Hanstein, 1984) and 2-azido-4-nitrophenol (NPA) (Hanstein et al., 1979) were synthesized as described. 2,4-Dinitro-6(2'-hydroxyethyl)phenol (HE-DNP) and 2,4-dinitro-6(2'-acetoxyethyl)phenol (AE-DNP) were prepared according to Dutton et al. (1953). 9-Amino-6-chlor-2-methoxyoacridine (ACMA) was a gift of Dr. E.P. Bakker, University of Osnabrück, and glutathione reductase (type III, yeast) was purchased from Sigma. All other reagents were of the highest commercially available purity.

RESULTS

The influence of the mode of energization on energy-dependent reactions can be studied most easily in the transhydrogenase reaction of submitochondrial particles because electron transport or ATP hydrolysis support this process equally well (Lee et al., 1964). Fig. 1 shows the effect of four structurally similar nitrophenols on transhydrogenation driven by succinate or ATP under otherwise identical conditions.

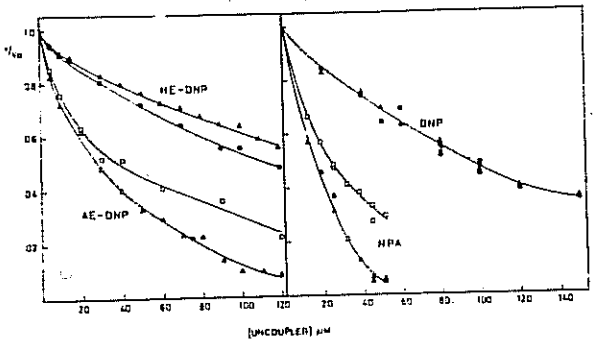

Fig. 1
Effect of nitrophenolic uncouplers on the rate of transhydrogenation supported by ATP hydrolysis (o) or succinate oxidation (Δ). The rates in the absence of uncouplers (Vo) were 0.202 ± 0.017 and 0.190 ± 0.016 µmoles NADH/min·mg submitochondrial protein in the presence of succinate and ATP, respectively.

It is seen that HE-DNP and, as shown before (van Dam & ter Welle, 1966), 2,4-dinitrophenol (DNP) do not significantly discriminate between the two energy sources, while AE-DNA and NPA lower the succinate-driven activity 3 to 5 times more effectively than the ATP-supported reaction. Among several possible reason for this unexpected finding, we investigated whether the generation of the protonmotive force or its use in transhydrogenation showed any dependence on the type of energy supply.

Membrane potential changes in submitochondrial particles can be spectroscopically monitored with potential-sensitive dye such as oxonol VI (Bashford & Smith, 1979). Fig. 2 shows a linear relationship between the spectral response of this probe and the steady-state membrane potential determined by the distribution of [14-C]-thiocyanate across the vesicle membrane.

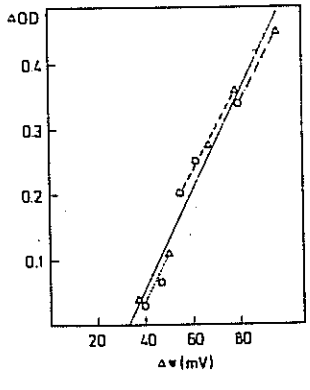

Fig. 2
The spectral response of oxonol VI in submitochrondrial particles as a function of the membrane potential maintained by ATP hydrolysis (o) or succinate oxidation (Δ). Increase of absorbance at 630 nm - 602 nm were measured in the absence (-·-·-) or presence of uncouplers (HE-DNP; - - -; AE-DNP:.....).

In this calibration, hydrogen peroxide and catalase were present to insure that sufficient oxygen was available to support succinate oxidation until sedimentation of the particles was complete. When the distribution of [14-C]-methylamine was determined, no significant pH-gradient could be detected even though uncoupler-sensitive quenching of ACMA fluorescence was observed. This confirms the finding of others that the membrane potential is the major component of the protonmotive force in this system (Branca et al., 1981). The effects of uncouplers on the membrane potential as measured by the oxonol response (Fig. 3) are very similar to those on transhydrogenation (Fig. 1).

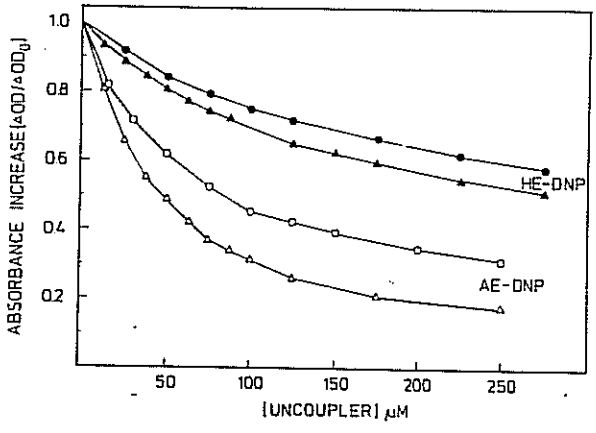

Fig. 3
Effect of the source of energization on the effectiveness of uncouplers in decreasing the membrane potential. The absorbance differences at 630 nm - 602 nm were normalized to the response in the absence of uncouplers. ATP (o), succinate (Δ).

With AE-DNP, the succinate-supported membrane potential is more easily diminished than that generated by ATP, while a much lesser effect is seen with HE-DNP.

The relationship of transhydrogenation rates with the membrane potential attenuated by AE-DNP and HE-DNP is seen in Fig. 4. It is apparent that neither source of energy nor uncoupler structure have any systematic influence. The curve in Fig. 4 is similar in shape to that found in the correlation of ATP synthesis rates and membrane potential (Sorgato et al., 1985). However, the slope at high membrane potential and the apparent abscissa intercept are much smaller than in phosphorylation, presumably because of the lower energy requirement of transhydrogenation (Earle & Fisher, 1980).

Delocalized protonic uncoupling in energy transduction requires that rate of energy linked reactions such as reverse electron flow, transhydrogenation and ATP synthesis depend, apart from their own kinetic constraints, only on the magnitude of the proton electrochemical gradient, and not on the mode by which it is changed. Therefore, the ratio of concentrations of two uncouplers inhibiting the reaction to a certain extent, e.g. 50%, should be the same, provided the two uncouplers decrease the proton-motive force in a similar way. Under less ideal circumstances, the isoeffective uncoupler concentration ratio can be expected to change monotonously as a function of the uncoupler sensitivity of the reaction. Data relevant to this postulate were obtained with HE-DNP and AE-DNP, two uncouplers with similar structure and molecular properties.

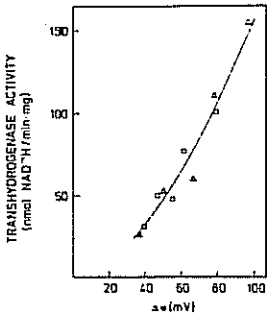

Fig. 4
The rate of energy-dependent transhydrogenation as a function of the membrane potential in submitochondrial particles. ATP (o), succinate (Δ). The curve represents a least-squares fit to the power curve y=ax(exp)b.

From the pK values of 4.2 and 4.6, and their octanol-water partition coefficients of 17 and 47, respectively (Schuermann, 1982), the ratio of concentrations necessary to achieve the same degree of uncoupling can be predicted. According to several correlations (Hansch et al., 1965), it should be in the range of 1.5 to 2.4. The actual ratios for a set of reactions are higher on the average, extending from 2.1 to 14.4 (Tab. 1). The large dispersion is in part due to the inclusion of values for mitochondria which, because of the larger contribution of the pH gradient, may not be strictly comparable to uncoupling in submitochondrial particles. Using only data obtained with the latter, the average uncoupler concentration ratio (4.1 ± 1.7) still has a large standard deviation. More importantly, as seen in Fig. 5, there is no correlation between the uncoupler concentration ratios and the susceptibility of energy-dependent reactions towards uncoupling.

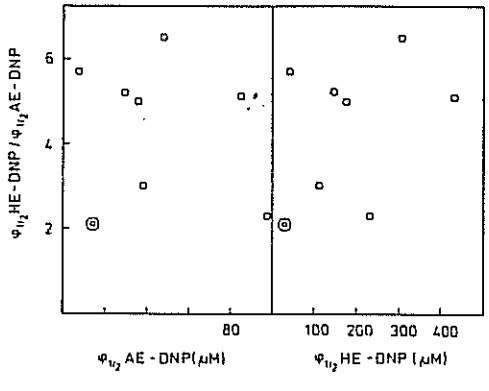

Fig. 5
Absence of correlation of the relative uncoupler effectiveness with the uncoupler concentrations necessary for half-maximal effects. AE-DNA: r^2=0.044: HE-DNP: r^2=0.214. Data for submitochondrial particles from Tab. 1

Table 1 Concentrations of uncouplers necessary for half-maximal effect on energy-dependent reactions

Reaction	vesicle	HE-DNP$^+$	AE-DNP$^+$	concentration ratio
reverse electron transport	SMP	40	7	5.7
transhydrogenation, succinate-supported	SMP	126	28	5.2
transhydrogenation, ATP-supported	SMP	112	38	3.0
ATP-Pi exchange	SMP	175	35	5.0
activation of ATPase	SMP	29	14	2.1
activation of succinate oxidase	SMP	30	14	2.1
ACMA fluorescence quenching ATP-supported	SMP	230*	98*	2.3
oxonol VI absorbance change succinate-supported	SMP	305	47	6.5
oxonol VI absorbance change ATP-supported	SMP	430	85	5.1
ADP/O ratio, 3-hydroxybutyrate-supported	mit	128	14	9.1
respiratory control, 3-hydroxy-butyrate oxidase	mit	173	12	14.4
respiratory control, succinate oxidase	mit	130	9	14.4

Abbreviations: SMP, beef heart submitochondrial particles;
mit, rat liver mitochondria;
$^+$concentration necessary for half-maximal inhibition or stimulation
*extrapolated values

DISCUSSION

In the present paper, quantitative relationships between uncoupling and uncoupler concentrations have been studied in order to test whether, under controlled conditions, attentuation of energy-dependent reactions depends solely on the decrease of the protonmotive force, or also on the source of energy or the type of reaction.

In energy-dependent transhydrogenation, two of the four uncouplers studied (AE-DNP, NPA) discriminate significantly between succinate and ATP as energy sources while HE-DNP and DNP do not (Fig. 1). It is apparent from the results in Fig. 3 that in these experiments the decrease of membrane potential mediated by uncouplers is influenced by the reaction used to generate the potential. The reason for this may be such as preferential inhibition of electron transport by AE-DNP and NPA. However, this is unlikely since there is considerable activation of succinate oxidase activity by uncouplers (Table 1), and more so with AE-DNP and NPA than with HE-DNP and DNP. Furthermore, this activation persists in all cases up to concentrations considerably higher than those used in this study (data not shown).

In the last several years, evidence has been presented that, in addition to the proton-electrochemical gradient across the inner mitochondrial membrane, directly coupled proton pumps (Westerhoff et al., 1984) or localized proton capacitances (Rottenberg, 1983; Rottenberg & Hashimoto, 1986) might be involved in energy transduction. Our data suggest that with some uncouplers, attentuations of the membrane potential appear to depend on the type of proton pump which provides energization (Fig. 3). This and the failure of uncouplers to inhibit a set of energy-dependent reactions in a quantitatively similar manner (Table 1, Fig. 5) are consistent with concepts of localized energy transfer modes which may be loosely coupled to the bulk phase process and therefore subject to similar, but quantitatively different uncoupling. As for the energy-dependent transhydrogenation, however, it is clear from the data in Fig. 4 that the rate of this reaction is independent of the primary energy source and can be related directly to the magnitude of the proton-motive force.

ACKNOWLEDGEMENTS

This work was supported by Grant Ha 1124, Deutsche Forschungsgemeinschaft.

REFERENCES

Bashford, C.L., & Smith, J.C., 1979. Meth. Enz., 55: 569-586.

Branca, D., Ferguson, S.J., & Sorgato, M.C., 1981. Eur. J. Biochem., 116: 341-346.

Dutton, G.G.S., Briggs, T.I., Brown, B.R., & Hillman, M.E.D., 1953. Can. J. Chem., 31: 685-687.

Earle, S., & Fisher, R.R., 1980. J. Biol. Chem., 255: 827-830.

Fujita, T., Iwasa, J., & Hansch, C., 1964. J. Am. Chem. Soc., 86: 5175-5180.

Hansch, C., Kiehs, K., & Lawrence, G.L., 1965. J. Amer. Chem. Soc., 87: 5770-5773.

Hanstein, W.G., & Hatefi, Y., 1974. Proc. Nat. Acad. Sci. USA, 71: 288-292.

Hanstein, W.G., Hatefi, Y., & Kiefer, H., 1979. Biochemistry, 18: 1019-1025.

Junge, W., Hong, Y.Q., Qian, L.P., & Viale, A., 1984. Proc. Nat. Acad. Sci. USA, 81: 3078-3082.

Kaschnitz, R.M., Hatefi, Y., & Morris, H.P., 1976. Biochim. Biophys. Acta, 449: 224-235.

Kell, D.B., & Westerhoff, H.V., 1985. In: Welch, C.R. (Ed.) Organized Multienzyme Systems, Academic Press, pp 63-139.

Kiehl, R., & Hanstein, W.G., 1984. Biochim. Biophys. Acta, 766: 375-385.

Lee, C.P., Azzone, G.F., & Ernster, L., 1964. Nature, 201: 152-155.

Löw, H., & Vallin, L., 1963. Biochim. Biophys. Acta, 69: 361-374.

Mitchell, P., 1985. In: Lee, C.P., Schatz, G., Dallner, G. (Eds.), Mitochondria and Microsomes, Addison-Wesley, pp 427-457.

Phelps, D.C., & Hanstein, W.G., 1977. Biochem. Biophys. Res. Commun., 79: 1245-1254.

Pullman, M.E., Penefsky, H.S., Datta, A., & Racker, E., 1960. J. Biol. Chem., 235: 3322-3329.

Pullman, M.E., 1967. Meth. Enz., 10: 57-60.

Rottenberg, H., 1979. Meth. Enz., 55: 547-569.

Rottenberg, H., 1983. Proc. Nat. Acad. Sci. USA, 80: 3313-3317.

Rottenberg, H., & Hashimoto, K., 1986. Biochemistry, 25: 1747-1755.

Schuermann, M., & Hanstein, W.G., 1980. EBEC Rep., 1: 243-244.

Schuermann, M., 1982. Thesis, University of Bochum, Medical Faculty.

Sorgato, M.C., Galliazzo, F., Valente, M., Cavallini, L., & Ferguson, S.J., 1982. Biochim. Biophys. Acta, 681: 319-322.

Sorgato, M.C., Lippe, G., Seren, S., & Ferguson, S.J., 1985. FEBS Letters, 181: 323-327.

van Dam, K., & ter Welle, H.F., 1966. In: Regulation of Metabolic Processes in Mitochondria, BBA Library, Elsevier, Amsterdam, 7: 235-245.

Westerhoff, H.V., Melandri, B.A., Venturoli, G., Azzone, G.F., & Kell, D.B., 1984. Biochim. Biophys. Acta, 768: 257-292.