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Steady-State Kinetics of the Overall Oxidative Phosphorylation Reaction in Heart Mitochondria. Evidence for Linkage of the Energy-Yielding and Energy-Consuming Steps by Freely Diffusible Intermediates and for an Allosteric Mechanism of Respiratory Control at Coupling Site 2

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Abstract

The three coupling segments of the respiratory chain of bovine heart mitochondria were examined individually by steady-state kinetic methods to determine whether or not freely diffusible intermediates occur between the energy-yielding and energy-consuming steps involved in the oxidative phosphorylation of extramitochondrial ADP. The principal method employed was the dual inhibitor technique, for which an appropriate model is provided. The results indicate that in accordance with the chemiosmotic theory the intermediate reactants that link the energy-yielding rotenone-sensitive (Site 1), cytochrome bc_1 (Site 2), and cytochrome aa_3 (Site 3) reactions of the respiratory chain to the energy-consuming ATP synthetase, AdN transport, and P_i transport reactions are freely diffusible (delocalized). Site 2 was found to differ from the others in regard to the mechanism by which the energy-linked respiratory chain reaction is controlled by the energy-consuming steps. Whereas the Site 1 and Site 3 respiratory chain reactions are controlled primarily by the thermodynamic mechanism of reaction reversal, the Site 2 respiratory reaction is controlled primarily by a kinetic mechanism in which an intermediate that links it to the energy-consuming steps inhibits it allosterically. From the effects of nigericin and valinomycin the allosteric intermediate appears to be the electrical component of the protonmotive force.

Key Words: Mitochondria; oxidative phosphorylation; steady-state kinetics; respiratory control; allosteric inhibition; dual inhibitor method; multienzyme reaction.

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Introduction

In previous studies of this nature (Stoner and Sirak, 1979; Stoner, 1984), three types of coupling relationships between different steps within the overall oxidative phosphorylation reaction were described. They concern the manner in which two steps affect each other in regard to how much they limit the rate of the overall reaction and depend on how the steps are coupled. The relationships were designated as sequential, nonsequential, and fixed, in which a change in the degree to which one is limiting results in a change in the opposite direction, in no change, and in a change in the same direction, respectively, in the degree to which the other is limiting. The sequential and nonsequential relationships are thought to occur only between individual reactions (i.e., between steps within the overall reaction that are linked by freely diffusible intermediate reactants), and the fixed relationship, although originally considered to occur only between steps within individual reactions (Stoner, 1984), can also occur between individual reactions (present communication).

In the first of the above studies the coupling relationships of the energy-yielding rotenone-, antimycin-, and cyanide-sensitive steps of the respiratory chain to the energy-consuming AdN[2] and P_i transport steps were determined under conditions where all the respiratory chain reactions were operating simultaneously. Under these conditions the coupling relationship of the antimycin-sensitive step to the AdN and P_i transport steps is nonsequential and the coupling relationships of the rotenone- and cyanide-sensitive steps to the AdN and P_i transport steps are sequential, although not to the degree one would expect if the reactions were linked in simple sequence (Stoner and Sirak, 1979). Since in phosphorylating mitochondria the rotenone-, antimycin-, and cyanide-sensitive steps are individual reactions (Stoner, 1984) and are known to be capable of driving the AdN and P_i transport reactions independently of one another, these coupling relationships might be expected to result from some sort of averaging of the true coupling relationships between the respiratory and energy-consuming reactions. Determination of the true coupling relationships between these reactions was the primary objective of this study. The approach was to pare down the overall reaction so that only one of the three energy-linked respiratory reactions was operational at a time and then to determine the coupling relationships of the one operational respiratory reaction to the energy-consuming steps.

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[2]Abbreviations used: AdN, adenine nucleotide; HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; $I_{0.5}$, concentration of inhibitor required to decrease the rate by one-half; S-13, 5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole.

In most cases the coupling relationships were determined by the very simple dual inhibitor technique used previously (Stoner, 1984). The original version of this technique as presented in model form by Baum *et al.* (1971) and more recently by Hitchens and Kell (1982a, 1983a, b) is seriously flawed owing to the facts that inhibition of the overall reaction by the second inhibitor was assumed to be linear in the absence of the first inhibitor and that the second inhibitor in the presence of the first was assumed to inhibit in accordance with the view that only one individual reaction can limit the rate of the overall reaction at a time. Linear inhibition of a steady-state multienzyme reaction having freely diffusible intermediates can occur only in the limiting case where the inhibited individual reaction is the only one limiting the rate of the overall reaction (Stoner and Sirak, 1979) and is unlikely to be found in natural multienzyme systems because such systems tend to be efficient in the use of enzymes and the most efficient use of enzymes occurs when the individual reactions are equally limiting (Waley, 1964; Stoner and Sirak, 1979).

An appropriate model for illustrating the dual inhibitor technique can be formulated on the basis of the simple multienzyme reaction

$$A \underset{k_2}{\overset{E_1,\ k_1}{\rightleftharpoons}} I \underset{k_4}{\overset{E_2,\ k_3}{\rightleftharpoons}} P \tag{1}$$

in which a substrate A is converted to a product P in two reversible enzyme-catalyzed reactions linked by a freely diffusible intermediate reactant I, and in which the concentrations of the reactants are sufficiently low in relation to the Michaelis constants that the reactions are in effect first-order reactions. It can be shown by very simple mathematical techniques (e.g., see Mahler and Cordes, 1971) that if A and P are assumed to be maintained constant (steady-state assumption) and that if the kinetics of the overall reaction are examined by the initial velocity method so that the concentration of P may be assumed to be essentially zero, the velocity v_0 of the uninhibited overall reaction (1) is given by the equation

$$v_0 = \frac{k_1 k_3 e_1 e_2 a}{k_2 e_1 + k_3 e_2} \tag{I}$$

in which e_1 and e_2 are the concentrations of the enzymes and a is the concentration of the substrate. Since in the dual inhibitor technique the effective concentration of one enzyme of a multienzyme reaction is specifically decreased in a graded fashion through inactivation by the second inhibitor, it is necessary now to obtain an expression for the velocity v_i of the overall reaction that takes into account the presence of this inhibitor. As shown in a similar derivation by Kröger and Klingenberg (1970), this can be

achieved simply by substituting into Eq. (I) an expression giving the fraction of the affected enzyme that is active. Thus we obtain the equation

$$v_i = \frac{k_1 k_3 e_1 \left(\frac{n_0 - n_i}{n_0}\right) e_2 a}{k_2 e_1 + k_3 \left(\frac{n_0 - n_i}{n_0}\right) e_2} \tag{II}$$

in which E_2 is arbitrarily taken to be the enzyme affected by the inhibitor, and n_i and n_0 are the inhibitor-inactivated and total concentrations of this enzyme, respectively. What is needed now is an expression giving the rate of the overall reaction in the presence of the inhibitor relative to the rate in the absence of the inhibitor. Such can be obtained simply by dividing Eq. (II) by Eq. (I). In doing so one can obtain the equation

$$\frac{v_i}{v_0} = \frac{\left(\frac{n_0 - n_i}{n_0}\right)\left(\frac{k_3}{k_2} + \frac{e_1}{e_2}\right)}{\left(\frac{n_0 - n_i}{n_0}\right)\frac{k_3}{k_2} + \frac{e_1}{e_2}} \tag{III}$$

Figure 1A presents three curves that were generated with the use of Eq. (III) on the assumptions that $k_2 = k_3$ and that the ratios of e_1 to e_2 are 0.1, 0.4, and 3 (upper, middle, and lower curves, respectively). The middle ("Control") curve is taken to be the inhibition curve of the second inhibitor obtained when a first inhibitor is absent, and the others are taken to indicate the kinds of shifts that can occur in the inhibition curve of the second inhibitor as a result of the overall reaction being inhibited by first inhibitors that bind at sites that are independent of the binding site of the second inhibitor. The curves are labeled according to the coupling relationships that would be indicated to exist between the steps inhibited by the first and second inhibitors.

A shift in the inhibition curve of the second inhibitor in the direction of the lower curve as a result of inhibition of the overall reaction by a first inhibitor would indicate a fixed coupling relationship between the inhibitor-sensitive steps and would be observed in the present example if the first inhibitor were to inhibit the E_2-catalyzed reaction. It is important to note that in the limiting case where inactivation of E_2 by the first inhibitor approaches 100%, the E_2-catalyzed reaction becomes the only one limiting the rate of the overall reaction, and inhibition by the second inhibitor in consequence becomes linear as though the overall reaction were a single-enzyme reaction. A shift in the curve in the direction of the upper curve would be indicative of a sequential coupling relationship between the inhibited steps and would be observed in the present example if the first inhibitor were to inhibit the

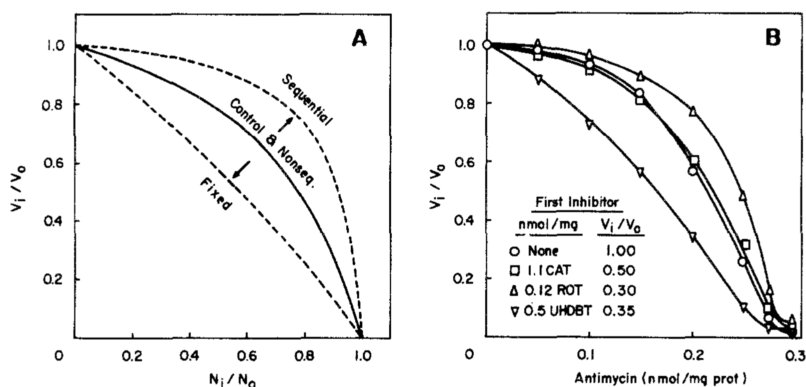

Fig. 1. Theoretical and experimental curves demonstrating the dual inhibitor method of determining coupling relationships between inhibitor-sensitive steps within steady-state multienzyme reactions. In part A the upper, middle, and lower curves were generated from Eq. (III) assuming $k_2 = k_3$ and e_1/e_2 ratios of 0.1, 0.4, and 3, respectively. In part B the curves were obtained through specific inhibition of the overall oxidative phosphorylation reaction in heart mitochondria oxidizing malate + pyruvate with O_2. Details concerning the composition of the incubation mixture are given in Materials and Methods. The mitochondria were preincubated for 2 min prior to the initiation of State 3 respiration by addition of ADP + hexokinase, and the average rate during the third minute following the addition of ADP was taken to be the steady-state rate. Antimycin, rotenone (ROT), and UHDBT were added initially. Carboxyatractyloside (CAT) was added 0.5 min after the ADP to avoid a slight but slow ADP-induced reversal of carboxyatractyloside inhibition that occurs when the concentration of ADP is as high as that employed (0.5 mM) and the inhibitor is added before the ADP. See the text for explanation of the curves. The v_0 rate in part B was 0.462 μatom O/min/mg mitochondrial protein.

E_1-catalyzed reaction. A lack of change in the inhibition curve of the second inhibitor would be indicative of a nonsequential ("Nonseq.") coupling relationship between the inhibited steps. This would mean that the inhibition by the first inhibitor has no apparent effect on the degree to which the E_2-catalyzed reaction is limiting. Such would be observed in the present example only if the first inhibitor were to inhibit the E_1- and E_2-catalyzed reactions equally, a circumstance that is not likely to be encountered in studies on real systems.

Figure 1B presents examples from the overall oxidative phosphorylation reaction of the three kinds of coupling relationships depicted in the model of Fig. 1A. The examples show that in phosphorylating heart mitochondria oxidizing malate + pyruvate with O_2, the coupling relationships of the antimycin-sensitive step to the rotenone-, carboxyatractyloside-, and UHDBT-sensitive steps are sequential, nonsequential, and fixed, respectively.

In regard to the model of Fig. 1A it should be noted that the concentration given for the second inhibitor refers only to the portion bound to the

enzyme the inhibitor inactivates and that the curves are thus independent of how tightly the inhibitor is bound. Owing to the great difficulty of obtaining this kind of information, inhibition curves are ordinarily formed by plotting total concentration of the inhibitor as in Fig. 1B. Consequently inhibition curves as ordinarily presented usually deviate considerably from those given by the model. The deviation in the case of antimycin inhibition is particularly small because the antimycin binding sites in heart mitochondria are very uniform in regard to affinity and bind antimycin so tightly under the conditions employed in the experiment of Fig. 1B that they become saturated at an antimycin concentration of only about twice the concentration of the binding site (Stoner and Sirak, 1979).

The use of total inhibitor concentrations in forming inhibition curves makes it difficult to predict magnitudes of shifts in the curves because this depends greatly on how tightly the inhibitor is bound (Stoner, 1984). Nevertheless, curves of this sort are very useful in that they give reliable indications of changes in the degree to which the inhibited reaction is limiting and of the directions of the changes. As shown previously (Stoner, 1984), such changes can be conveniently followed by noting the effect of inhibition by one of the inhibitors on the $I_{0.5}$ of the other.

Since the purpose of the inhibitors in the dual inhibitor technique is to change specifically the degrees to which particular steps of a multienzyme reaction are limiting, the inhibitors can be dispensed with if other means of doing this are available (e.g., see Stoner and Sirak, 1979). In this regard it is important to note that although uncoupling agents are inhibitors, they are not of the kind that one would ordinarily use in the dual inhibitor technique. Hitchens and Kell (1983a, b) recently used uncouplers in this manner in studies on light-driven ATP synthesis in bacterial chromatophores. Since uncouplers inhibit ATP synthesis by catalyzing a reaction that competes with the ATP synthetase reaction for the intermediates that link this step to the energy-yielding reactions, the results of such studies are somewhat difficult to interpret. Consequently uncouplers probably should be used only under conditions where the normal energy-consuming steps are inactive and be considered as catalysts of substitute energy-consuming steps rather than as inhibitors. In the present study the uncoupler S-13 was used in this manner and compared with the catalysts of the normal energy-consuming steps. The results show that the true coupling relationships between the energy-yielding and energy-consuming steps are sequential at Sites 1 and 3 and fixed at Site 2. However, as previously noted (Stoner, 1984), the fixed relationships at Site 2 appear to come about by allosteric mechanisms rather than by coupling mechanisms that are fundamentally different from those at Sites 1 and 3.

Materials and Methods

Mitochondria were isolated from slaughterhouse bovine hearts according to a slight modification of a Nagarse procedure (Stoner and Sirak, 1968). Homogenates were prepared in a medium containing 250 mM sucrose, 0.5 mM K-EGTA, 0.05 mM K-EDTA, 10 mM imidazole-HCl (pH 7.4 at 0°C), and 0.2 mg of Nagarse proteinase per gram of tissue. The mitochondria were washed twice and suspended finally in a medium containing 250 mM sucrose and 5 mM K-Pipes (pH 7.0). Use of the mitochondria was commenced immediately after isolation.

The first coupling segment of the respiratory chain (Site 1) was assayed using K-malate + K-pyruvate (5 mM each) as the oxidizable substrate and K-ferricyanide (1.5 mM) as the terminal electron acceptor in an incubation mixture (3 ml) that also contained 140 mM sucrose, 10 mM glucose, 10 mM K-Pipes (pH 6.5), 1 mM $MgCl_2$, 0.1 mM K-EGTA, 2 nmol myxothiazol/mg mitochondrial protein, 2 mM KCN-HCl (from a fresh 0.5 M stock solution, pH 8.0), 0.5 mg BSA/ml, 12 μM ubiquinone-1, and 0.25 mg mitochondrial protein/ml. When phosphorylating conditions were used, the concentration of K-Pipes was decreased to 5 mM, and 5 mM K-phosphate (pH 6.5), 0.5 mM Na-ADP, and 2.5 units of yeast hexokinase/ml were included in the incubation mixture. Under these conditions of high ADP concentration the amount of hexokinase used is saturating (Stoner and Sirak, 1979). The overall reaction was initiated after 5 min of incubation at 30°C by adding the ferricyanide. The rate was determined from the average rate of ferricyanide reduction over a 100-sec period beginning 1 min after the reaction was started. The rate of ferricyanide reduction was determined in an Aminco DW-2 spectrophotometer from the wavelength difference 440–490 nm using an absorbance coefficient of 0.586 $mM^{-1} \times cm^{-1}$.

The second coupling segment (Site 2) was assayed using K-succinate (10 mM) as the oxidizable substrate and K-ferricyanide (1.5 mM) as the terminal electron acceptor. Other conditions were as described for the Site 1 assay except that myxothiazol, BSA, and ubiquinone-1 were omitted from the incubation mixtures and 8 nmol rotenone/mg mitochondrial protein was included.

The third coupling segment (Site 3) was assayed using 7.5 mM K-ascorbate (+0.75 mM TMPD) as the oxidizable substrate and O_2 as the terminal electron acceptor. Other conditions of incubation were as described for Site 2 except that the cyanide and rotenone were omitted from the incubation mixtures, antimycin at 2.5 nmol/mg mitochondrial protein was included, and the concentration of the mitochondria was decreased to 0.05 mg protein/ml. The concentration of oxygen in the incubation mixtures (8 ml) was monitored under rapid stirring in a closed, thermostated (30°C)

reaction chamber (Stoner and Sirak, 1973). In determining the $I_{0.5}$ for cyanide, the TMPD and cyanide were initially excluded from the incubation mixture and the mitochondria were preincubated for 1 min prior to adding the cyanide (KCN) and for 3 min prior to initiating respiration by adding the TMPD. The average rate during the 5th min of incubation following the addition of TMPD was taken to be the steady-state rate of respiration. Stable TMPD stock solutions were prepared as previously described (Stoner, 1984) from TMPD-HCl which had been recrystallized twice according to LuValle *et al.* (1948).

In the assays of Site 1 + Site 2, K-malate + K-pyruvate (5 mM each) was the oxidizable substrate and K-ferricyanide (1.5 mM) the terminal electron acceptor. Other conditions were as described for the Site 2 assay except that rotenone was excluded from the incubation mixtures. In the Site 2 + Site 3 assays, K-succinate (10 mM) was the oxidizable substrate and O_2 the terminal electron acceptor. Other conditions were as described for the Site 3 assay except that antimycin was excluded from the incubation mixtures, rotenone at 8 nmol/mg mitochondrial protein was included, and the concentration of mitochondria was 0.25 mg protein/ml. The Site 1 + Site 2 + Site 3 assay procedure was the same as that of the Site 2 + Site 3 assay except that K-malate + K-pyruvate was the oxidizable substrate and rotenone was omitted from the incubation mixtures. The presence of other constituents of the incubation mixtures and deviations from the conditions outlined above are presented with the data.

Rotenone, aurovertin, and UHDBT were quantitated spectrophotometrically in ethanol using absorbance coefficients ($mM^{-1} \times cm^{-1}$) of 17.0 at 295 nm for rotenone (Büchi *et al.*, 1961), 42.7 at 367.5 nm for aurovertin (Baldwin *et al.*, 1964), and 12.2 at 287 nm for UHDBT in ethanol containing 0.1 mM acetic acid (Trumpower and Haggerty, 1980). HQNO was dissolved in ethanol but was quantitated spectrophotometrically in 1 mM aqueous NaOH using a mM absorbance coefficient of 9.45 at 346 nm (Cornforth and James, 1956). Ubiquinone-1 was quantitated according to Schatz and Racker (1966).

Yeast hexokinase (Type F-300), oxidizable substrates, Pipes, ADP, rotenone, and HQNO were obtained from Sigma Chemical Company (St. Louis). 3′-*n*-Hexylcarboxin was generously donated by G. A. White (Agriculture Canada Research Center, London, Ontario), aurovertin by C. L. Baldwin (Dow Chemical Company, Zionsville, Indiana), carboxyatractyloside by S. Luciani (Univeristy of Padua), and S-13 by P. C. Hamm (Monsanto Company, St. Louis). Myxothiazol, UHDBT, and ubiquinone-1 were obtained from J. S. Rieske (Ohio State University) and were originally gifts from W. Trowitzsch (Gesellschaft für Biotechnologische Forschung, Braunsweig), B. L. Trumpower (Dartmouth Medical School), and Hoffmann-LaRoche (Basel), respectively.

Results

Preliminary Studies

A number of preliminary studies were conducted to assure that the assays were reliable. As was noted above, the Site 1 assays were carried out with malate + pyruvate as the oxidizable substrate and ferricyanide as the terminal electron acceptor. The energy-yielding sequence of the overall Site 1 reaction thus included Krebs cycle acid transport and oxidation reactions in addition to the energy-linked rotenone-sensitive NADH dehydrogenase reaction of the respiratory chain. Since the NADH dehydrogenase is capable of interacting with redox reactants only at the inner side of the inner membrane (Lawford and Garland, 1974) and ferricyanide does not penetrate the inner membrane (Klingenberg and Buchholz, 1970), it was neccessary to provide a redox intermediate that is capable of shuttling electrons between the dehydrogenase and ferricyanide. Ubiquinone-1 was found to work very well for this purpose, and the activity in the Site 1 system was absolutely depenent on its presence. The activity was subject to complete inhibition by rotenone, indicating that only the energy-linked pathway was involved (Schatz and Racker, 1966; Lawford and Garland, 1972; DiVirgilio and Azzone, 1982). The concentration of ubiquinone-1 employed ($12\,\mu$M) was adequate for maximum activity. The inclusion of BSA in the incubation mixtures was found to stimulate the activity very noticeably and to prevent a decline during the period of measurement.

The energy-yielding sequence of the overall Site 2 reaction included the succinate transport and succinate dehydrogenase reactions in addition to the energy-linked cytochrome bc_1 reaction. A number of inhibitors of these reactions were employed. As in a previus study (Stoner, 1984), phenylsuccinate was used as an inhibitor of the succinate transport reaction, and a potent analog of carboxin was used as an inhibitor of the succinate dehydrogenase reaction. In the case of the bc_1 reaction several inhibitors (antimycin, HQNO, myxothiazol, UHDBT, funiculosin, and mucidin) were used in the preliminary experiments, and the major effects of bc_1 inhibitors reported below were found not to be specific for a particular bc_1 inhibitor. In acquiring data for presentation, UHDBT was the inhibitor of choice because it equilibrates very rapidly with its binding site, is bound loosely enough that changes in its $I_{0.5}$ are easy to detect (Stoner, 1984), and is capable of inhibiting the overall Site 2 reaction nearly completely. The use of antimycin was unsafe because this inhibitor is inactivated by ferricyanide when unbound (Walter and Lardy, 1964) and, as was learned in the preliminary studies, is inactivated significantly also by cyanide (2 mM).

In the case of the overall Site 3 reaction, the conditions were such that the energy-linked cytochrome aa_3 reaction was likely in effect the only

individual reaction of the energy-yielding sequence of steps. Thus, under the conditions of high ascorbate and TMPD concentrations employed, the TMPD is maintained largely in the reduced state and cytochrome *c* bound to the cytochrome aa_3 complex probably goes through several redox cycles before dissociating from the complex (Hill and Nicholls, 1980). For the reasons given previously (Stoner, 1984), the inhibitor of choice for this reaction was cyanide, which was found capable of inhibiting the Site 3 reaction virtually completely.

Determination of the True Coupling Relationships between the Energy-Yielding and Energy-Consuming Steps

Table I presents the results of six experiments that were designed to reveal the true coupling relationships of the individual energy-linked respiratory chain reactions to the ATP synthetase, AdN transport, and S-13-catalyzed reactions. Rotenone, UHDBT, and cyanide were employed as second inhibitors of the respiratory reactions at Sites 1, 2, and 3, respectively, and aurovertin as a first inhibitor of the ATP synthetase reaction and carboxyatractyloside as a first inhibitor of the AdN transport reaction. The effects of the uncoupler were determined only in nonphosphorylating mitochondria, as were also the individual effects of nigericin and valinomycin.

Examination of the results obtained with phosphorylating mitochondria reveals that inhibition at the level of the energy-consuming steps by either aurovertin or carboxyatractyloside results in the $I_{0.5}$ of the respiratory inhibitor increasing in the cases of Sites 1 and 3 and decreasing in the case of Site 2, indicating the existence of sequential coupling relationships at Sites 1 and 3 and fixed coupling relationships at Site 2. It may be noted that in all cases as the level of inhibition of the energy-consuming step was increased, the $I_{0.5}$ of the respiratory inhibitor approached that obtained with mitochondria incubated in the absence of phosphate and phosphate acceptor. From this it is evident that regardless of which of the energy-consuming steps is inhibited, results similar to those obtained with aurovertin and carboxyatractyloside can be anticipated.

As may be been from the data obtained with S-13, results that are consistent with those described above are obtained when an uncoupler-catalyzed reaction is substituted for the normal energy-consuming steps. Thus, as the S-13-catalyzed reaction is made less rate limiting by increasing the concentration of the uncoupler, the $I_{0.5}$ of the respiratory inhibitor decreases in the cases of Sites 1 and 3, indicating that the respiratory reactions become more limiting, and increases in the case of Site 2, indicating that the respiratory reaction becomes less limiting. In the cases of Sites 1 and 2, the rate of the overall reaction increases with increase of S-13 concentration up

Table I. Determination of the True Coupling Relationships between the Energy-Yielding and Energy-Consuming Steps through Separate Examinations of the Individual Coupling Segments[a]

		Site 1		Site 2		Site 3	
Experiment[b]	Additions[c]	Ferricyanide reduction (μmol/min/mg)	$I_{0.5}$ rotenone (nmol/mg)	Ferricyanide reduction (μmol/min/mg)	$I_{0.5}$ UHDBT (nmol/mg)	Oxygen reduction (μatom/min/mg)	$I_{0.5}$ cyanide (μM)
1	ADP + P_i + HK	0.77	0.08	0.75	0.75	1.12	2.3
	+ 0.2 AV	0.66	0.10	0.72	0.67	1.06	2.5
	+ 0.4 AV	0.25	0.35	0.51	0.57	0.80	3.3
	+ 1.2 CAT	0.72	0.09	0.69	0.63	1.06	2.6
	+ 1.4 CAT	0.51	0.12	0.63	0.55	0.95	2.9
	+ 1.6 CAT	0.24	0.41	0.48	0.52	0.82	3.2
	None	0.13	2.3	0.47	0.52	0.65	3.1
2	None	0.12	1.4	0.48	0.60	0.68	3.3
	0.1 μM NIG	0.11	1.1	0.42	0.68	0.78	3.2
	0.1 μM VAL	0.26	0.28	0.38	3.1	0.82	1.9
	0.001 μM S-13	0.14	0.76	0.51	0.67	0.72	2.9
	0.01 μM S-13	0.39	0.15	0.87	1.0	1.11	2.3
	0.1 μM S-13	0.56	0.09	0.69	1.8	1.44	1.1
	1 μM S-13	0.43	0.11	0.57	2.2	1.53	0.9
	ADP + P_i + HK	0.65	0.09	0.85	0.74	1.19	2.8

[a] HK = hexokinase; AV = aurovertin; CAT = carboxyatractyloside; NIG = nigericin; VAL = valinomycin.
[b] The data given for the individual coupling sites in experiments 1 and 2 were also obtained in separate experiments.
[c] The concentrations of aurovertin and carboxyatractyloside are given as nmol/mg mitochondrial protein.

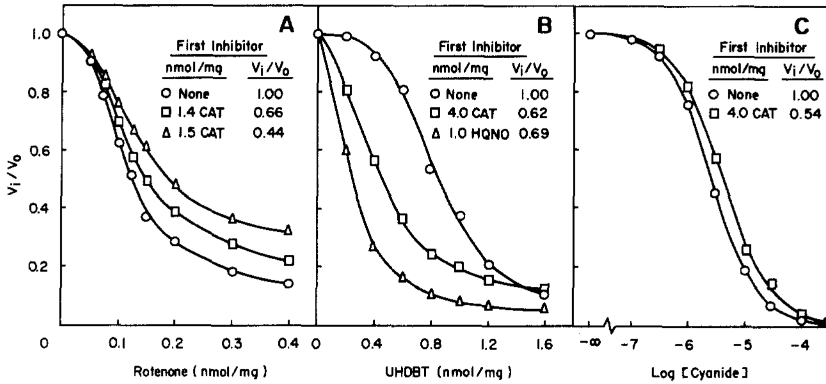

Fig. 2. Graphic illustration of the coupling relationships between the energy-yielding and energy-consuming steps at Coupling Sites 1, 2, and 3 as determined by the dual inhibitor method. The data of parts A, B, and C were obtained with a single mitochondrial preparation according to the assay procedures described in Materials and Methods for Sites 1, 2, and 3, respectively. In parts B and C the concentration of carboxyatractyloside (CAT) used was saturating. The v_0 rates in parts A and B were 0.96 and 0.86 μmol ferricyanide reduced/min/mg protein, respectively, and the v_0 rate in part C was 1.07 μatom O/min/mg protein.

to a point, which occurs at a higher concentration of the uncoupler in the case of Site 1 than in that of Site 2, and then decreases. As previously indicated (Stoner, 1984), the inhibition of succinate oxidation (Site 2) at high concentrations of an uncoupler is likely due primarily to inhibition of succinate dehydrogenase by oxalacetate as a result of the uncoupler decreasing the level of intramitochondrial ATP. Evidence indicating that the uncoupler-induced inhibition in fact occurs at the succinate dehydrogenase level is given below. In the case of Site 1, the inhibition seems to set in as a result of an uncoupler-induced leakage of AdN's from the mitochondria and of consequent slow turnover of GTP (Olson and Allgyer, 1972) and inhibition of the Krebs cycle at the level of the succinate thiokinase reaction. Presumably inhibition of malate + pyruvate oxidation occurs at the level of the pyruvate dehydrogenase reaction as a result of *S*-succinyl-CoA accumulation and consequent depletion of CoASH (LaNoue *et al.*, 1972). In accordance with this, the inhibition can be largely prevented by preincubating the mitochondria with parapyruvate, a specific inhibitor of the α-ketoglutarate dehydrogenase reaction (Montgomery and Webb, 1956), strongly enhanced by agents that promote the loss of AdN's from mitochondria [e.g., P_i (Meisner and Klingenberg, 1968) and PP_i (Stoner and Sirak, 1976)], and reversed by ADP or ATP.

Nigericin has little affect on the coupling segment reactions of nonphosphorylating mitochondria. Nevertheless, the effects shown (Table I) proved to be very reproducible. Valinomycin has relatively large effects. In

the cases of Sites 1 and 3, it has effects similar to those of the uncoupler, whereas in the case of Site 2 it inhibits the overall reaction, often quite strongly (50%), and invariably induces a very large increase in the $I_{0.5}$ of UHDBT, indicating that it makes the bc_1 reaction very much less limiting.

To provide a more graphic view of the difference in coupling relationships between Site 2 and Sites 1 and 3, an experiment of the kinds summarized in Table I is presented in graphic form in Fig. 2. It can be seen that in the cases of Sites 1 and 3, carboxyatractyloside inhibition clearly shifts the curve of the respiratory chain inhibitor toward higher concentrations of the inhibitor, whereas in the case of Site 2 carboxyatractyloside inhibition clearly shifts the inhibition curve of the respiratory chain inhibitor toward a lower concentration of the inhibitor. In addition it can be seen that in the Site 2 system, inhibition by carboxyatractyloside is somewhat less effective than inhibition by the bc_1 inhibitor HQNO in decreasing the $I_{0.5}$ of UHDBT.

Investigations of the Mechanism of the Fixed Coupling Relationships at Site 2

As was noted in the Introduction, a fixed coupling relationship between two inhibitor-sensitive steps could mean that the two steps are part of the same individual reaction and thus that they are not linked by a freely diffusible intermediate. Such an explanation for the fixed relationships at Site 2 was immediately difficult to accept because it seemed highly unlikely that the mechanisms of coupling at one coupling site would differ so drastically from those at the others, particularly in view of the lack of evidence for such in other kinds of studies. It became particularly difficult to accept when it was learned that even the hexokinase- and S-13-catalyzed reactions, which are known to be linked to the bc_1 reaction by freely diffusible intermediates, have a fixed coupling relationship to the bc_1 reaction. Consequently it seemed likely that the mechanisms of coupling at Site 2 do not differ from those at the other sites, but that inhibition of the energy-consuming steps results in an increase in the level of a freely diffusible product of the bc_1 reaction that links the bc_1 reaction to at least one of these steps and is more inhibitory to the bc_1 reaction by an allosteric mechanism than by the thermodynamic mechanism of reaction reversal.

To test this view and the possibility that something was wrong with the approach, the predictions of the dual inhibitor technique in respect to the relationship of the energy-consuming steps to the succinate transport and succinate dehydrogenase reactions in the overall Site 2 reaction were tested. Since it is known (Stoner, 1984) that sequential coupling relationships exist between the bc_1, succinate transport, and succinate dehydrogenase reactions, one could clearly expect these relationships to be sequential. As shown in Table II, this expectation proved to be correct.

Table II. Coupling Relationships of the Energy-Consuming Steps to the Succinate Transport and Succinate Dehydrogenase Steps in the Overall Site 2 Reaction[a]

Additions[b]	Ferricyanide reduction (μmol/min/mg)	$I_{0.5}$ 2-PS (mM)	$I_{0.5}$ 3′nHC (μM)	$I_{0.5}$ UHDBT (nmol/mg)
ADP + P_i + HK	0.85	3.3	0.22	0.84
+ 1.4 CAT	0.61	6.5	0.30	0.59
+ 1.6 CAT	0.49	9.6	0.37	0.56
None	0.45	9.9	0.56	0.58
0.001 μM S-13	0.50	9.9	0.39	0.73
0.01 μM S-13	0.77	4.0	0.14	1.2
0.1 μM S-13	0.62	5.4	0.12	2.1
1 μM S-13	0.53	6.2	0.13	2.3

[a] HK = hexokinase; CAT = carboxyatractyloside; 2-PS = phenylsuccinate; 3′nHC = 3′-n-hexylcarboxin.
[b] The concentration of carboxyatractyloside is given as nmol/mg mitochondrial protein.

The experiment of Table II is of interest also in regard to the mechanism by which high levels of uncoupler inhibit the overall Site 2 reaction. Thus the results show that as the concentration of S-13 is increased and as the rate of the overall Site 2 reaction increases and then decreases in response, the $I_{0.5}$ of the succinate dehydrogenase inhibitor decreases to a low value as the rate of the overall reaction reaches a maximum and then does not undergo appreciable further change as the rate decreases. The lack of further change in the $I_{0.5}$ of the succinate dehydrogenase inhibitor as the rate declines strongly suggests that the succinate dehydrogenase reaction becomes the only step that is significantly limiting as the rate of the overall reaction is increased to a maximum by uncoupling. Thus if the succinate dehydrogenase reaction were essentially the only one limiting the overall reaction, it might be expected to behave kinetically as though it were in a single-enzyme system, and in single-enzyme systems the $I_{0.5}$'s of inactivators of the enzyme do not change as the level of activity is changed by means that do not interfere with the binding of the inactivator.

Since an unnatural electron acceptor was used in the Site 2 system, it seemed possible that the allosteric inhibition does not normally occur, but that one or both of the components of the protonmotive force alter the conformation of the bc_1 complex such that reduction of ferricyanide by the complex is more difficult. A simple way to investigate this possibility would be to examine the coupling relationships of the energy-linked respiratory reactions with two of the respiratory reactions operating simultaneously. Since the Site 1 and Site 3 respiratory reactions have the same coupling relationship to the energy-consuming steps, and since simultaneous opera-

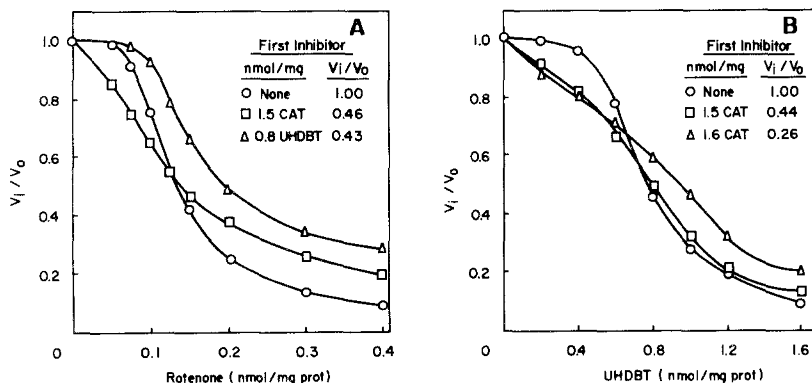

Fig. 3. Coupling relationships between the energy-yielding and energy-consuming steps at Sites 1 and 2 as determined by the dual inhibitor method under conditions of simultaneous operation of these coupling segments. The data of parts A and B were obtained with the same mitochondrial preparation, and in both cases the assay conditions were as described for Site 1 + Site 2 in Materials and Methods. CAT = carboxyatractyloside. The v_0 rates in parts A and B were 0.83 and 0.88 μmol ferricyanide reduced/min/mg protein, respectively.

tion of Sites 1 and 2 would involve the use of the unnatural electron acceptor at Site 2 and simultaneous operation of Sites 2 and 3 would not, one could expect to see indications of averaging of a sequential coupling relationship with a fixed relationship in the case of the Site 1 + Site 2 system but not in the case of the Site 2 + Site 3 system if the fixed relationships come about as a result of using the unnatural acceptor.

Figure 3 presents the results of a dual inhibitor experiment on a system in which Sites 1 and 2 were simultaneously operational, and Fig. 4 presents the results of a similar experiment on a system in which Sites 2 and 3 were simultaneously operational. It can be seen that very similar results were obtained with the two systems and that in all cases partial inhibition of the overall reaction by carboxyatractyloside shifted the inhibition curve of the respiratory inhibitor to the left at low levels of inhibition and to the right at high levels of inhibition. These results strongly suggest that the fixed relationships observed at Site 2 exist in the natural system.

It is interesting to note that the $I_{0.5}$'s of the respiratory chain inhibitors in the Site 1 + Site 2 and Site 2 + Site 3 systems change very little as a result of carboxyatractyloside inhibition and that ambiguous results could be obtained if the $I_{0.5}$'s were used as the criterions of change in the degrees to which the individual respiratory reactions are limiting. For example, in Fig. 3B the $I_{0.5}$ of UHDBT is essentially unchanged by carboxyatractyloside inhibition at one high level but is substantially increased as the degree of

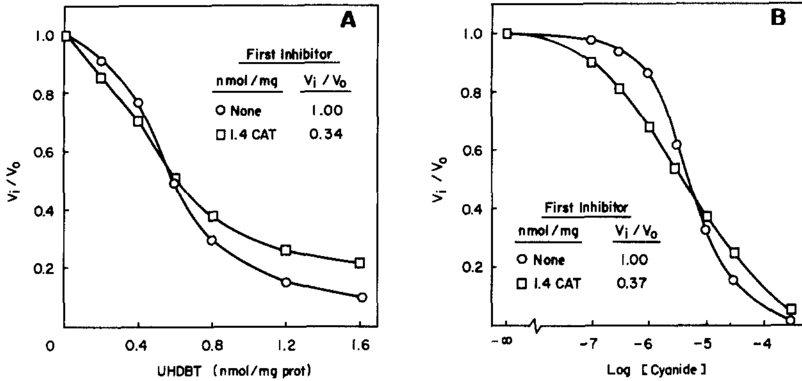

Fig. 4. Coupling relationships between the energy-yielding and energy-consuming steps at Sites 2 and 3 as determined by the dual inhibitor method under conditions of these coupling segments operating simultaneously. The data of parts A and B were obtained with a single mitochondrial preparation, and in both cases the assay conditions were as described for Site 2 + Site 3 in Materials and Methods. In the experiment of part A the mitochondria were preincubated for 2 min prior to initiating State 3 respiration by adding ADP + hexokinase, and the average rate during the third minute following the addition of ADP was taken to be the steady-state rate. In the experiment of part B the mitochondria were preincubated in the absence of oxidizable substrate for 1 min prior to the addition of cyanide and for 2 min following the addition of cyanide. The average rate of respiration during the 5th minute of incubation following the addition of succinate was taken to be the steady-state rate. The v_0 rates in parts A and B were 0.395 and 0.365 μatom O/min/mg protein, respectively.

carboxyatractyloside inhibition is increased. As can be seen from Fig. 2, this sort of problem is not encountered when the three coupling segments are examined individually, and it can be seen from Fig. 3A that it is also not encountered in determining the coupling relationship between the respiratory chain reactions in the Site 1 + Site 2 system.

Discussion

On the Coupling Relationships between the Energy-Yielding and Energy-Consuming Steps

The results of this study show that the true coupling relationships between the energy-yielding and energy-consuming steps of the overall oxidative phosphorylation reaction are sequential at Sites 1 and 3 and fixed at Site 2. However, the fixed coupling relationships at Site 2 appear not to indicate the absence of freely diffusible intermediates because fixed relationships are indicated even in cases where it is certain that such intermediates exist. Consequently it seems likely that the Site 2 reactions are linked to each other

in the same manner as those at Sites 1 and 3 and that the fixed relationships come about through an allosteric mechanism. According to this interpretation the bc_1 reaction is linked to the energy-consuming steps by freely diffusible intermediates but exhibits fixed rather than sequential coupling relationships to these steps because at least one of the intermediates inhibits the bc_1 reaction more effectively by an allosteric mechanism than by the mechanism of reaction reversal. To comprehend this fully, one must see clearly the difference between product inhibition by the mechanism of reaction reversal and product inhibition by an allosteric mechanism in relation to the fixed and sequential coupling relationships.

Product inhibition by reaction reversal is associated with an increase in the concentration ratio of product to substrate and thus with the reaction concerned operating closer to thermodynamic equilibrium. When it occurs in an individual reaction of a simple sequence as a result of inhibition by other means elsewhere in the sequence, it is associated with the individual reaction concerned being less rate limiting in the overall reaction catalyzed by the sequence and is the mechanism by which the sequential coupling relationship comes about. It does not come about through inactivation of the catalyst and occurs regardless of whether or not the reaction concerned is catalyzed. In contrast, product inhibition by allosteric mechanisms occurs through inactivation of the catalyst and thus resembles the kinds of inhibitions induced by specific inhibitors in this study. Consequently, when it occurs in a multienzyme reaction consisting of individual reactions, one might expect it to be associated with the individual reaction concerned operating farther from thermodynamic equilibrium and thus with the individual reaction being more limiting in the overall reaction.

Consideration of what might be expected of the different kinds of product inhibition in regard to coupling relationships is best achieved by examining simple model reactions of the type

$$A \underset{}{\overset{E_1}{\rightleftharpoons}} I_1 \overset{E_2}{\rightleftharpoons} I_2 \overset{E_3}{\rightleftharpoons} I_3 \overset{E_4}{\rightleftharpoons} P \qquad (2)$$

In this reaction a substrate A is converted to a product P by way of four enzyme-catalyzed reactions linked by the freely diffusible intermediate reactants I_1, I_2, and I_3. The concentrations of the substrate and product are assumed to be maintained constant by continuous flux of A in and P out. That product inhibition by the mechanism of reaction reversal is associated with the sequential coupling relationship can easily be seen by considering what would happen if we were to decrease slightly the effective concentration of one of the enzymes, say E_3, by adding to the system an inactivator of this enzyme. It is clear that product inhibition by reaction reversal would occur in each of the two upstream reactions and would result in these reactions operating closer to thermodynamic equilibrium. Of course the inhibition

would result also in the downstream reaction operating closer to equilibrium, but through a decrease in the concentration of the substrate rather than through an increase in the concentration of the product. Now let us assume that one of the upstream reactions, say the E_2-catalyzed reaction, is subject to allosteric inhibition by its product I_2 in addition to inhibition by the mechanism of reaction reversal. Since allosteric inhibition occurs through enzyme inactivation and since inactivation at E_3 would increase the concentration of I_2, inactivation would occur also at E_2. In addition, since it would be possible that I_2 binds very tightly to the allosteric site on E_2 and in doing so inactivates E_2 completely, I_2 could be a very effective inactivator of E_2 and thus could inhibit the E_2-catalyzed reaction much more by the allosteric mechanism than by the mechanism of reaction reversal. If such were the case, a fixed coupling relationship would exist between the E_2- and E_3-catalyzed reactions, and, since the level of I_2 would increase also as a result of inhibition of other downstream reactions, a fixed coupling relationship would exist also between the E_2- and E_4-catalyzed reactions. From this it is clear that allosteric inhibition of the bc_1 reaction by a product that drives the energy-consuming reactions can account for the observation that all the reactions of the energy-consuming sequence, including the hexokinase reaction, have a fixed coupling relationship to the bc_1 reaction.

It should be noted that for a particular degree of inhibition of overall reaction (2), inactivators that inactivate E_2 by increasing the concentration of I_2 could never be as effective as a nonproduct inactivator of E_2 in making the E_2-catalyzed reaction more limiting. This follows from the fact that the E_2-catalyzed reaction is linked to the downstream reactions by freely diffusible intermediates, a consequence of which is that inhibition downstream would always tend to make the E_2-catalyzed reaction less limiting through the mechanism of reaction reversal and thus less sensitive to inactivators of E_2. From this it is evident that the observation of Fig. 2B that for a particular degree of inhibition of the overall Site 2 reaction, carboxyatractyloside inhibition is less effective than HQNO inhibition in making the bc_1 reaction more limiting would be expected even if the allosteric inhibitor of the bc_1 reaction were extremely effective.

On the Nature of the Allosteric Intermediate in the Overall Site 2 Reaction

It is now virtually certain that the energy-yielding and energy-consuming steps of the overall oxidative phosphorylation reaction are linked by the $\Delta\psi$ and ΔpH components of the protonmotive force predicted by the chemiosmotic theory. To gain some idea as to which of these components is more important in mediating the allosteric inhibition of the bc_1 reaction, the individual effects of nigericin and valinomycin on the $I_{0.5}$'s of a number of bc_1 inhibitors in the Site 2 assay were determined under conditions of absence of

phosphate and phosphate acceptor. As indicated in Table I for UHDBT, nigericin was found to have little affect whereas valinomycin was found invariably to increase the $I_{0.5}$'s of the bc_1 inhibitors very markedly. Since in K^+ media valinomycin tends to dissipate $\Delta\psi$ and to result in an increase in ΔpH, these observations suggest that the allosteric intermediate is $\Delta\psi$. This is in agreement with the observation of Rich and Clark (1982) and Papa *et al.* (1983) that electron flow in the bc_1 reaction is controlled primarily by the $\Delta\psi$ component.

Since valinomycin inhibits the overall Site 2 reaction (Table I), one might suppose that it increases the $I_{0.5}$'s of bc_1 inhibitors by inhibiting the succinate transport reaction or the succinate dehydrogenase reaction. However, preliminary studies suggest that such an explanation would not be correct because (1) valinomycin inhibition results in these reactions becoming less limiting rather than more limiting, and (2) for a given degree of inhibition of the overall reaction, valinomycin increases the $I_{0.5}$'s of bc_1 inhibitors very much more than do inhibitors of the succinate transport and succinate dehydrogenase reactions. At present little more is known concerning the mechanism of the inhibition by valinomycin.

On the Mechanisms of Respiratory Control

Respiratory control in mitochondria, as originally defined, is the respiratory inhibition that occurs when mitochondria having an ample supply of oxidizable substrate are deprived of phosphate or phosphate acceptor (Lardy and Wellman, 1952). Although not generally recognized, there are two kinds or mechanisms of respiratory control between which a distinction should be made: kinetic and thermodynamic. The control is kinetic when it occurs as a result of mechanical limitation, and thermodynamic when it occurs as a result of the backward and forward rates of the reaction approaching one another. Since enzymes are "mechanical" and when deprived of substrate are inactive, the control that occurs as a result of depriving the overall oxidative phosphorylation reaction of ADP or P_i is clearly of the kinetic kind. On the other hand, the control that occurs as a result of a saturating amount of ADP becoming phosphorylated in the presence of a saturating amount of P_i is clearly of the thermodynamic kind.

It should be noted that in deciding which of the two kinds of control limits a reaction, the reaction must be specified. Consider, for example, a system of tightly coupled mitochondria incubated in the presence of a high concentration of oxidizable substrate and absence of ADP and P_i. If the reaction under consideration were the overall oxidative phosphorylation reaction, the control would be primarily kinetic owing to mechanical limitation at the levels of the AdN and P_i transport reactions. However, if we were to concern ourselves with control at the level of one of the individual

reactions upstream from the AdN and P_i transport reactions other than the bc_1 reaction, the control would be primarily thermodynamic. Now if we were to add ADP and P_i to this system, the level of control overall would decrease, the rate of the overall reaction would increase, and of the control remaining, that of the AdN and P_i transport reactions would be less kinetic and more thermodynamic and that of the upstream reactions other than the bc_1 would be less thermodynamic and more kinetic. From this rendering it is evident that although both serve to limit reactions, the two kinds of control differ greatly in regard to propagation among individual reactions and in regard to rate limitation of individual reactions as determined by the degrees of disequilibrium at which they are operating.

Respiratory control is normally not complete, and this is usually attributed to the presence of energy-dissipating processes such as proton cycling (Nicholls, 1974; Stucki, 1976), Ca^{2+} cycling (Stucki and Ineichen, 1974), P_i cycling (Stoner and Sirak, 1978), and ATPase activity (Chao and Davis, 1972; Masini *et al.*, 1983). Although there is little question that such processes occur and that when they do they decrease the degree of respiratory control, the marked difference in the degree of control among the coupling sites (Hatefi *et al.*, 1961) suggests that the degree of control is decreased significantly also by "molecular slipping," a looseness of coupling characterized by the transport of electrons through a coupling site without the transport of protons (Pietrobon *et al.*, 1981). In the experiments of Table I, for example, the ratios of the rates observed in the presence and absence of ADP + P_i + hexokinase were 5.7, 1.7, and 1.7 at Sites 1, 2, and 3, respectively. This difference does not appear to have occurred as a result of the differences in the conditions of incubation or of the differences in the energy spans. Consequently, it seems likely to have occurred largely as a result of a difference among the sites in tightness of coupling.

The results of this study seem consistent with the conclusions that respiratory control at Sites 1 and 3 occurs primarily through Δp inhibition by the thermodynamic mechanism of reaction reversal and that respiratory control at Site 2 occurs primarily through $\Delta\psi$ inhibition by an allosteric (kinetic) mechanism. Since it is reasonable to suppose that at Site 2 the allosteric inhibition occurs in addition to product inhibition by the thermodynamic mechanism, and since the degree of respiratory control at Site 2 is about equal to that at Site 3 and much less than that at Site 1, one might suppose that product inhibition by the mechanism of reaction reversal is particularly ineffective at Site 2. However, it seems likely that if such were the case, one could expect the bc_1 reaction to be relatively irreversible in tightly coupled mitochondria. Since in mitochondria of this sort the bc_1 reaction is approximately equally as reversible as the rotenone-sensitive reaction and much more reversible than the cytochrome aa_3 reaction (Klingenberg, 1964),

this is clearly not the case. Therefore it seems reasonable to suggest that the apparent deficiency of thermodynamic control at Site 2 is due to molecular slipping under conditions of high Δp and that the allosteric mechanism may have come to exist as a result of this coupling deficiency.

It is interesting to note that in consequence of there being fixed coupling relationships between the energy-consuming steps and the bc_1 reaction and sequential coupling relationships between the energy-consuming steps and the succinate transport and succinate dehydrogenase reactions, respiratory control at Site 2 increases when the overall Site 2 reaction is inhibited by a bc_1 inhibitor and decreases when it is inhibited by a succinate transport or succinate dehydrogenase inhibitor. This difference has been noted also by Pietrobon *et al.* (1981) and Mandolino *et al.* (1983) and taken by the former as evidence for molecular slipping in the redox-driven proton pumps. The observations of Ernster and Nordenbrand (1974), suggesting that only inhibition of the energy-consuming steps decreases the degree of coupling between the energy-yielding and energy-consuming steps, suggest that uncoupling events such as molecular slipping occur mostly when Δp is very high, and thus that the above inhibitor-induced changes in the degree of respiratory control are not indicative of changes in the degree of coupling.

On the Mechanism of Energy Coupling

The observations of this study indicating the presence of freely diffusible intermediates between the energy-yielding and energy-consuming steps strongly suggest that in accordance with the implications of the postulates of Mitchell (1966), Δp is generated between the bulk phases separated by the coupling membrane and is thus delocalized within individual mitochondria. Although Δp is generally accepted as the link between the energy-yielding and energy-consuming steps, the implication that it is delocalized is widely questioned (e.g., see Baccarini-Melandri *et al.*, 1977; Williams, 1978; Kell, 1979; Hitchens and Kell, 1982a, b, 1983a, b; Ferguson and Sorgato, 1982; Zoratti *et al.*, 1982; Mandolino *et al.*, 1983; Westerhoff *et al.*, 1984). Some of the skepticism has come from dual inhibitor studies similar to those reported here. Thus Hitchens and Kell (1982b) (see also Venturoli and Melandri, 1982) found that the light-driven phosphorylation reaction carried out by chromatophores from *Rhodopseudomonas capsulata* becomes more sensitive to oligomycin as the reaction is inhibited by antimycin and becomes more sensitive to antimycin as the reaction is inhibited by oligomycin. This indicates the existence of a fixed coupling relationship between the antimycin- and oligomycin-sensitive steps and, as noted above, could mean that these steps are linked by a fixed (localized) intermediate rather than by a freely diffusible (delocalized) one. However, in view of the considerable similarity between bacterial chromatophores and mitochondria in regard to antimycin-

and oligomycin-sensitive reactions (Dutton *et al.*, 1975; Crofts and Wood, 1978; Baccarini-Melandri *et al.*, 1981) and of the virtual certainty that the similar fixed coupling relationship observed in mitochondria comes about by an allosteric mechanism, it seems likely that the fixed relationship in the case of the chromatophore reaction also comes about by an allosteric mechanism. A simple way to find out would be to link the chromatophore reaction to the hexokinase reaction and determine whether or not a fixed coupling relationship exists also between the antimycin-sensitive and hexokinase reactions.

Another observation made by the above workers and taken by them as evidence for Δp being localized is that inhibition of the chromatophore phosphorylation reaction with either antimycin or an inhibitor of the ATP synthetase step increases the sensitivity of ATP synthesis to inhibition by uncoupling agents (Hitchens and Kell, 1983a, b). As was noted in the Introduction, this kind of dual inhibitor experiment is quite complex owing to one of the inhibitors inhibiting the indicator reaction by catalyzing a reaction that competes with the indicator reaction for one of its substrates. Nevertheless such experiments can be interpreted, and the above results seem best interpreted by assuming that the ATP synthetase and uncoupler-catalyzed reactions compete for freely diffusible Δp. With this assumption one could expect the application of a specific inhibitor of the ATP synthetase reaction to result in this reaction being less effective in competing with an uncoupler-catalyzed reaction for Δp and thus in less uncoupler being required to achieve a particular degree of inhibition of the ATP synthetase reaction. The observation that inhibition by the electron transport inhibitor antimycin also results in uncouplers being more effective can be explained by the fact that a fixed coupling relationship exists between the antimycin-sensitive and ATP synthetase reactions and thus that antimycin is in effect also an inhibitor of the ATP synthetase reaction.

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