MINIREVIEW

Use of Top-Down Elasticity Analysis to Identify Sites of Thyroid Hormone-Induced Thermogenesis (43852B)

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Abstract. **Top-down elasticity analysis is a novel extension of top-down metabolic control analysis. It has provided researchers with a theoretical and practical platform upon which quantitative analyses of the sites of action of hormones and drugs can be based. This approach is easy to apply and involves dividing up the metabolic system in question into two or three blocks of enzyme reactions around an intermediate between the blocks of reactions. The kinetic response of each block to the intermediate is measured in the steady state *in situ* by determining the flux through the block at different measured intermediate concentrations. The intermediate can be manipulated by titrating the other blocks with suitable inhibitors or activators. Then, to determine which blocks of reactions are quantitatively the most important in terms of any change in the flux rate of the system, a quantitative comparison of the titration curves from the experimental preparations is made with those of the control preparations. In this minireview we will examine, as an example, the use of top-down elasticity analysis for the quantitative identification of the important sites of action of thyroid hormones on oxidative phosphorylation in hepatocytes. The experimental results show that approximately 50% of the change in resting oxygen consumption in hepatocytes from hypothyroid and hyperthyroid rats (compared with euthyroid controls) is attributable to changes in the rate of the mitochondrial proton leak; the remaining 50% is accounted for by changes in nonmitochondrial- and ATP turnover-dependent oxygen consumption in hypothyroid and hyperthyroid hepatocytes, respectively.** [P.S.E.B.M. 1995, Vol 208]

Before the advent of metabolic control analysis, knowledge of the control of fluxes through metabolic pathways and of the control of concentrations of metabolites was nonquantitative. Metabolic control was studied primarily by isolating individual enzymes, assessing their kinetics *in vitro* and devising an intuitive model of the control of the pathway of interest. As a result, it was thought that certain steps in metabolic pathways were dominant and rate limiting. Qualitative analyses of this type could then be corroborated by determining whether effector molecules, such as hormones, that were known to change the kinetics of the supposed rate-limiting step also changed the flux through the entire pathway. Sites of control in a metabolic pathway were often recognized by "cross-overs" in the concentration of metabolites

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that occur in response to the addition of an effector. The cross-over sites were identified as those where substrates were accumulated and products depleted when the reaction was slowed by the addition of the effector, or as those where substrates were depleted and products accumulated when the reaction was accelerated by the addition of the effector. These methods were clearly labour intensive and, in the end, yielded only qualitative data which provided little insight into the situation *in vivo*.

Based on such approaches for analyses of metabolic control, various theories have been proposed for the control of cellular respiration and oxidative phosphorylation, and they have been reviewed by Brand and Murphy (1). Predominant among the theories are those in which extramitochondrial adenine nucleotide phosphorylation potential (ΔG_p, ATP and ADP concentrations, ATP/ADP ratio, etc.) is the main controller of respiration. Although these theories are correct in identifying phosphorylation status as an important factor, they fail to take into account the control of respiration by all of the reactions that supply reducing equivalents to the electron transport chain (e.g., tricarboxylic acid cycle, oxidation of fatty acids).

Metabolic Control Analysis and the Development of the Top-Down Elasticity Analysis Approach

Metabolic control analysis was originally developed by Kacser and Burns (2), and Heinrich and Rapoport (3), and the underlying theory has been reviewed several times (1, 4–6). Simply put, metabolic control analysis is a quantitative approach that is of both theoretical and practical use for determining the control structure of a metabolic system. Because top-down elasticity analysis is an extension of metabolic control analysis and the data generated in a top-down elasticity analysis provide all of the information needed to complete a control analysis of the metabolic system being studied, we will first outline the principles underlying metabolic control analysis before describing the top-down elasticity analysis approach.

Metabolic control analyses provide quantitative information about the importance of reactions, or blocks of reactions, in the control of the flux through a metabolic pathway and in the control of the concentration of metabolites within that pathway. This information is mathematically conveyed in the form of flux control and concentration control coefficients, respectively. Theoretically, there are two approaches which can be taken when analyzing a system using metabolic control analysis. The experimental application of the traditional approach to metabolic control analysis (2, 3) depends on the ability to manipulate individual enzyme activities using inhibitors or genetic approaches, or the ability to do relatively large numbers of enzyme kinetics analyses. The aim of this approach is to determine the flux control coefficients of *individual enzymes* over the flux through a metabolic pathway. By repeatedly applying this approach, the flux control coefficients of more and more enzymes within the pathway can be determined. However the applicability of this traditional approach has been restricted to particular enzymes and systems because of the necessity for, and limited availability of, specific inhibitors for enzymes acting within the system being studied.

The aim of the second approach to metabolic control analysis, the top-down approach, is to determine the distribution of control between two or three blocks of reactions comprising a system (7). In contrast to the traditional approach, the top-down approach generates flux control coefficients and concentration control coefficients for blocks of enzyme reactions in a metabolic pathway. This approach involves conceptually dividing the metabolic pathway into two or three blocks of reactions around one of its intermediates. An intermediate is a component of the system which is produced by one or more pathway(s) and is consumed by the subsequent pathway(s). For example, if the purpose was to investigate how the kinetics of ATP-producing and -consuming reactions were affected by a certain drug, then cytoplasmic ATP concentration would be an appropriate intermediate to measure.

To determine the distribution of control within the system being studied, the concentration of the intermediate is manipulated by titrating the blocks of reactions with inhibitors or activators. The control coefficients of the two or three blocks are then derived from measurements of the dependence of the fluxes on the magnitude of the intermediate. The latter are described numerically in the form of *elasticity coefficients* which therefore indicate the overall kinetics of the block of reactions to changes in the amount of the intermediate under the prevailing experimental conditions. In more formal terms, the elasticity of a block of reactions is defined as the fractional change in the flux through the block of reactions that is caused by an infinitesimal fractional change in the concentration of the intermediate. The equations that are used to calculate control coefficients from the experimentally derived elasticities can easily be derived and are published (8).

In both the top-down approach and the traditional approach, the sum of all flux control coefficients for the overall pathway is 1.0. A flux control coefficient having a value near 1.0 means that the block of reactions for which that coefficient was determined has almost exclusive control over the flux through the pathway. Similarly a block of reactions that has a low flux control coefficient has little control over the flux. High and low values of concentration control coefficients indicate high and low amounts of control, respectively, by blocks of reactions over the concentra-

tion of the intermediate. The top-down approach can then be repeated around different intermediates to give a progressively fuller picture of the distribution of control within the overall pathway.

The requirements for the application of top-down metabolic control analysis to a system are minimal. First, the intermediate measured must be representative of the intermediate that the system actually uses. In other words, the intermediate must not be channeled, compartmented, or diffusion limited. The second requirement is that the blocks of reactions affect each other solely through the concentration of the intermediate. Thus, the activity of one branch in the system cannot affect the activity of another branch except through changes in the amount of the intermediate. For example, in the oxidative phosphorylation system shown in Figure 1, the only means through which the substrate oxidation reactions can alter the activity of the phosphorylating reactions is by a change in the mitochondrial membrane potential. There is no 'cross talk' between the pathways within the system—otherwise, changes in the producers of the intermediate might alter the rate of the consumers of the intermediate through routes other than through the intermediate and the results of a control analysis would be invalid. In practice, when the structure of the pathway is well understood, whether this requirement is met may be known. If it is not known, it can be tested by measuring the elasticity of the producers of the intermediate to changes in the amount of the intermediate by altering the concentration of the intermediate using various inhibitors of the reactions that consume the intermediate. The elasticities determined from the various approaches should not be different; if they are, then the reactions that consume the intermediate affect the flux through the producers of the intermediate by a means other than through the concentration of the intermediate. In this case, a more complex analysis would be required, or another metabolite in the system should be chosen as the intermediate.

An advantage of the top-down approach of great practical use is that the inhibitors or activators used to determine the kinetics of the branches do not necessarily need to have known sites of action, as is the case with the traditional approach. For example, if a given inhibitor of gluconeogenesis, such as β-mercaptopicolinate, is used to inhibit the flux through the phosphorylation pathway, it is not necessary to know the exact site(s) of inhibition. However, it is necessary that it act only within the intended pathway and not affect the overall kinetics of the other pathways of the system (i.e., the substrate oxidation and proton leak pathways).

The top-down approach has wide applicability. It can be applied to the study of metabolic systems in organelles, whole cells, and intact organs. Moreover, as was recently described by Brown (9), metabolic control analysis can also be used at the level of the whole body, under certain conditions, to determine the quantitative importance of various organs for the synthesis and disappearance of metabolites (e.g., ketone bodies). Finally, the application of the approach is not limited by the ability to measure the intermediate, because it is possible to determine the overall kinetics of the pathways through calculations based on flux rates which have been determined under various experimental conditions (6).

A limitation of metabolic control analysis which is a common characteristic of experimental approaches is that the results provide information on the control of a system and apply only to the experimental conditions under which the components of the system were measured. For example, the quantitative importance of thyroid hormone-induced changes in the phosphorylation branch of the oxidative phosphorylation system towards a change in the flux through the system in

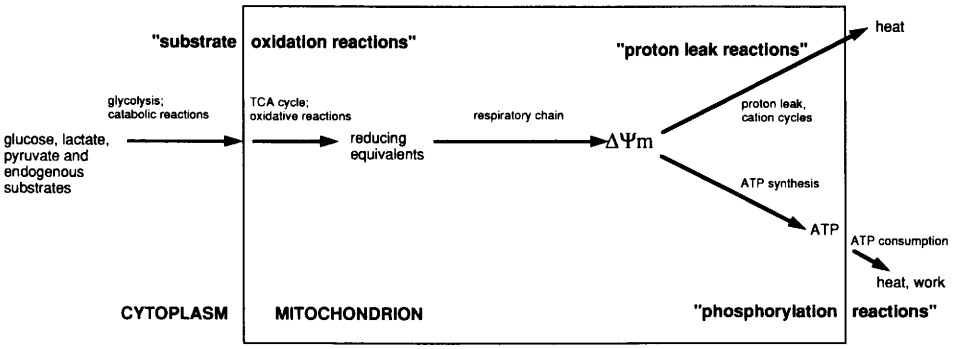

Figure 1. Oxidative phosphorylation in hepatocytes. Mitochondrial membrane potential ($\Delta\Psi_m$) is produced by the "substrate oxidation reactions" which consist of all of the steps from added glucose, lactate, and pyruvate, and from endogenous substrates to $\Delta\Psi_m$. In liver mitochondria, the substrate oxidation reactions consist of only the TCA cycle, mitochondrial oxidative reactions, and the respiratory chain. Then $\Delta\Psi_m$ is consumed by the "phosphorylation reactions" which include mitochondrial ATP production and all cellular ATP-consuming reactions, or it is consumed by the "proton leak reactions" which include the leak of protons and any cation cycles across the mitochondrial inner membrane. In liver mitochondria, the phosphorylation reactions consist of only those reactions which contribute to mitochondrial ATP synthesis (i.e., the phosphate carrier, adenine nucleotide translocator and the ATP synthase).

intact cells would be expected to be different from that in isolated mitochondria as the components of the phosphorylation system differ between intact cells and isolated mitochondria (Fig. 1, and discussed in more detail below).

Top-Down Elasticity Analyses

While metabolic control analyses identify the sites of control within metabolic pathways, top-down elasticity analyses are used for the identification of the sites of regulation within metabolic pathways by hormones and other external effectors (10). Beyond their use in the identification of the sites of action of thyroid hormones in mitochondria (11–13) and in hepatocytes (14–16), top-down elasticity analyses have been used successfully to investigate the sites of action of glucagon (17), and butylated hydroxyanisole (18) in mitochondria and of fatty acids in hepatocytes (19).

Top-down control analysis has been used to determine how oxidative phosphorylation is controlled. For our analyses, we have defined the oxidative phosphorylation system in hepatocytes as shown in Figure 1. In this system, the intermediate is mitochondrial membrane potential ($\Delta\Psi_m$), and the three surrounding blocks of reactions include the substrate oxidation, proton leak, and the phosphorylation blocks of reactions. Top-down control analyses using rat hepatocytes have shown that ATP turnover reactions (flux control coefficients of 0.4–0.5) and substrate oxidation reactions (flux control coefficients of 0.3–0.4) exert most of the control over oxygen consumption (i.e., the flux through the system), while the remainder of the control is through the proton leak (flux control coefficients of 0.2–0.3) (14, 20). Concentration control coefficients derived in the same studies showed that the blocks of reactions that are most important in the control of $\Delta\Psi_m$ are the substrate oxidation block of reactions followed by the phosphorylation and leak blocks of reactions.

To complete a top-down elasticity analysis, a top-down control analysis is carried out in the presence and absence of some effector that changes the steady state flux through the system. If the site of action of the effector is in the block of reactions that produces the intermediate, then the overall elasticity curve for this block will be changed; if the site of action is after the intermediate, then the elasticity curve of the consumers of the intermediate will be changed. Thus, for the oxidative phosphorylation system shown in Figure 1, in order to determine the site(s) of action of a hormone, the elasticity curves of the three blocks of reactions in the presence and absence of the hormone are compared. For example, as shown in Figure 2, if the hormone increases the steady state oxygen consumption rate of cells by changing the kinetic response of the $\Delta\Psi_m$ producers (i.e., the substrate oxidation

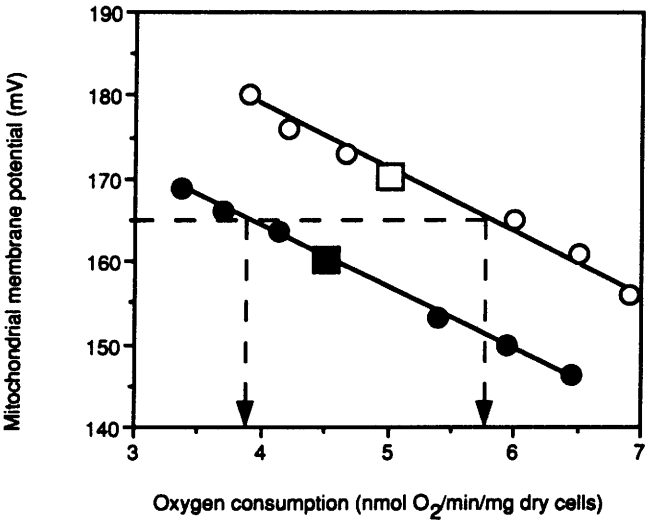

Figure 2. Hypothetical example of a hormonal effect on the substrate oxidation reactions in cells. The filled symbols represent the titration points in the absence of the hormone. The open symbols represent the titration points in the presence of the hormone. The squares represent the resting mitochondrial membrane potential and oxygen consumption values (i.e., in the absence of any inhibitors and uncouplers). The overall kinetics of the $\Delta\Psi_m$ producers (i.e., the substrate oxidation reactions) are determined by titrating, for example, with either oligomycin or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). When oligomycin is added to the cell suspensions the $\Delta\Psi_m$ consumers (i.e., the phosphorylation and leak reactions) are inhibited through the inhibition of ATP synthase; the amount of the intermediate (i.e., $\Delta\Psi_m$) increases and oxygen consumption is inhibited. This is shown by the data points that rise to the left of each resting value. When the uncoupler FCCP is added to the cell suspensions the $\Delta\Psi_m$ producers are stimulated (by the dissipation of $\Delta\Psi_m$); the amount of the intermediate (i.e., $\Delta\Psi_m$) decreases and oxygen consumption is stimulated. This is shown by the data points that fall to the right of each resting value. Because the elasticity curves show that at any given $\Delta\Psi_m$ (e.g., 165 mV) oxygen consumption is approximately 1.5-fold greater in the presence of the hormone than in its absence, the results show that the hormone stimulates the $\Delta\Psi_m$ producers by this factor.

reactions), then the elasticity curve representing the $\Delta\Psi_m$ producers will be different in the presence of the hormone compared with that in the absence of the hormone. Thus, at a given $\Delta\Psi_m$, oxygen consumption that is attributable to the activity of the $\Delta\Psi_m$ producers is *x*-fold greater in the presence of the hormone. Then by repeating the elasticity analysis around different intermediates within the block(s) of reactions that were identified as being affected by the hormone, the site of action can gradually be narrowed down to one or two of the individual reactions within that block. As well as identifying the reactions or blocks of reactions that are affected, the data from top-down elasticity analyses can be used to determine the quantitative importance of the changes in the activity of the reactions. Therefore, one can state, for example, that of the change in the respiration rate of cells which is induced by the addition of a certain hormone, *y*% is due to a change in this block of reactions, and *z*% is due to that block of reactions. Finally, because top-down elastic-

ity analyses provide all of the data that are needed to complete a full metabolic control analysis of the system, a comparison of the control coefficients in the presence and absence of the hormone will determine whether the hormone changes the distribution of control over the flux through the system or over the concentration of the intermediate.

The Application of Top-Down Elasticity Analysis to Identify the Important Sites of Thyroid Hormone Action in Oxidative Phosphorylation

Despite extensive research into the effects of thyroid hormones on pathways of cellular energy expenditure, there is no consensus as to the quantitatively important sites of action of thyroid hormones in bioenergetic pathways. The literature in this area is extensive and has been reviewed recently by Soboll (21). Several studies have supported the theory that thyroid hormones predominantly affect the mitochondrial dehydrogenases and components of the electron transport chain (22–26). The results of other studies have identified the adenine nucleotide transporter (25, 27–30), the ATP synthase (24, 31), and the extramitochondrial ATPases, Na,K-ATPase and Ca-ATPase (see Ref. 32 and 33), as the important targets of thyroid hormones. Increases in protein synthesis and gluconeogenesis (34), as well as in lipogenesis (35), are also thought to account for thyroid hormone-induced changes in oxidative phosphorylation and cellular oxygen consumption. The findings of some relatively recent studies have shown that changes in the rate of the leak of protons across the mitochondrial inner membrane contribute to the altered rate of oxygen consumption in mitochondria and cells in hypothyroid and hyperthyroid rats. The proton leak rate is increased during State 4 respiration in rat liver mitochondria during hyperthyroidism and decreased during hypothyroidism (11, 12). These effects of thyroid hormones on the mitochondrial proton leak have also been observed in intact hepatocytes from hypothyroid rats (14, 36) and hyperthyroid rats (14).

The application of top-down elasticity analysis to this area of metabolic research has provided some quantitative answers to questions about the importance of particular reactions in the oxidative phosphorylation system towards thermogenic changes in liver mitochondria and hepatocytes from hypothyroid and hyperthyroid rats.

Studies Using Rat Liver Mitochondria: The Importance of the Mitochondrial Proton Leak. Top-down elasticity analysis was first used by Hafner *et al.* (11) to investigate the effects of thyroid hormones on State 4 respiration in liver mitochondria from hypothyroid, euthyroid, and hyperthyroid rats. Since there is no phosphorylation during State 4 respiration, the system was an unbranched two-part system comprising the electron transport chain and the proton leak. The proton leak titration curves were different in hypothyroid and hyperthyroid mitochondria compared with the proton leak titration curve in euthyroid mitochondria.

The top-down approach was then used to investigate the effects of thyroid hormones on oxidative phosphorylation in State 3 liver mitochondria from hypothyroid rats and euthyroid controls, (13) and from hyperthyroid rats and euthyroid controls (15). To ascertain whether the substrate oxidation block of reactions or the phosphorylation block of reactions, or both, were responsible for the hypothyroid-induced decrease in State 3 oxygen consumption, Hafner *et al.* (13) studied the kinetics of these blocks of reactions in mitochondria. In State 3, the rate of the proton leak is very low and the control of the leak over mitochondrial oxygen consumption rate by the leak is close to zero (13, 37); thus, the contribution of the change in the proton leak that dominates effects on State 4 respiration is insignificant for the change in mitochondrial oxygen consumption rate in State 3 liver mitochondria from hypothyroid rats.

To determine whether the substrate oxidation block of reactions was affected by hypothyroidism, oxygen consumption and mitochondrial membrane potential ($\Delta\Psi_m$) were titrated with uncouplers and the titration curves from liver mitochondria of hypothyroid and euthyroid rats were compared. If hypothyroidism inhibited the substrate oxidation block of reactions, then the plot of mitochondrial oxygen consumption rate against $\Delta\Psi_m$ obtained from the uncoupler titration of euthyroid mitochondria would lie above the plot obtained with hypothyroid mitochondria, such that at any given $\Delta\Psi_m$ the oxygen consumption rate would be higher in the euthyroid mitochondria than in the hypothyroid mitochondria. However, the results of Hafner *et al.* (13) indicated that there was no difference in the kinetics of the substrate oxidation block of reactions in hypothyroid and euthyroid mitochondria—showing that any changes in the respiratory chain do not significantly contribute to the decreased State 3 oxygen consumption rate in hypothyroid mitochondria.

The possibility that the phosphorylation block of reactions was affected by hypothyroidism was investigated using malonate titrations of oxygen consumption and $\Delta\Psi_m$, and the titration curves from hypothyroid and euthyroid mitochondria were compared. The $\Delta\Psi_m$-consuming reactions were found to be inhibited by hypothyroidism. As the rate of the proton leak is minimal in State 3 respiration, the $\Delta\Psi_m$-consuming reactions in these mitochondria mainly comprise the adenine nucleotide translocator, ATP synthase, and the phosphate transporter reactions.

The results thus showed that the $\Delta\Psi_m$ consumers

are inhibited and the $\Delta\Psi_m$ producers are unchanged in hypothyroid mitochondria compared with euthyroid mitochondria. It was concluded that the decrease in State 3 oxygen consumption in hypothyroid mitochondria compared with controls is totally attributable to a decrease in the rate of the phosphorylation block of reactions. Based on the earlier finding that the phosphate transporter has a very low flux control coefficient over State 3 respiration rate (38), it was reasoned (13) that the phosphate transporter was likely an unimportant site of thyroid hormone action. Changes in the adenine nucleotide translocator and ATP synthase activities are quite likely since thyroid hormone-induced changes in both the kinetics of the adenine nucleotide translocator and the total amount of ATP synthase have been widely reported (24, 25, 27–31). Overall, the results in hypothyroid mitochondria show that State 4 oxygen consumption is decreased as a result of a decrease in the amount of the proton leak and that State 3 oxygen consumption is decreased as a result of a decrease in the activity of the $\Delta\Psi_m$ consumers which most likely include the ATP synthase and the adenine nucleotide translocator.

What are the sites of action of thyroid hormones when respiration is intermediate between State 3 and 4? Using the top-down elasticity analysis approach we recently investigated the effects of hyperthyroidism on oxidative phosphorylation in rat liver mitochondria respiring at State 3 and 4, and intermediate states of respiration (15). The experimental approach was essentially identical to that described above for hypothyroid mitochondria. The kinetics of the proton leak were altered in hyperthyroid State 4 mitochondria compared with euthyroid controls. The results showed that at any given oxygen consumption rate, $\Delta\Psi_m$ was lower in hyperthyroid mitochondria than in euthyroid control mitochondria. Thus the results confirmed those of Hafner *et al.* (11) in hypothyroid, euthyroid, and hyperthyroid State 4 mitochondria. The results, however, extended those of Hafner *et al.* (11) by describing the kinetics of the two other branches of the system, the substrate oxidation and the phosphorylation reactions. At respiration rates intermediate between State 3 and 4, the overall kinetics of the substrate oxidation reactions were determined. The data showed that at high respiration rates (approaching State 3 respiration) the kinetics of the substrate oxidation reactions differed between hyperthyroid and euthyroid mitochondria. The kinetics of the phosphorylation block of reactions to $\Delta\Psi_m$ showed that there was no difference in the overall kinetics of these reactions in hyperthyroid and euthyroid control mitochondria. Thus any changes in the activities of the phosphorylation reactions under our experimental conditions were quantitatively insignificant in terms of the increased oxygen consumption rates of hyperthyroid mitochondria compared with controls. These unexpected results from mitochondria are discussed further below in light of the results obtained from intact hepatocytes. Thus the results of this top-down elasticity analysis of the effects of hyperthyroidism on oxidative phosphorylation in rat liver mitochondria identified the mitochondrial proton leak and the respiratory chain as the important sites of action of thyroid hormones in mitochondria from hyperthyroid rats compared with controls.

Studies Using Rat Hepatocytes: The Importance of the Mitochondrial Proton Leak and of Non-mitochondrial Oxygen Consumption and ATP Consumers. Resting oxygen consumption rates of hepatocytes from hyperthyroid rats and hypothyroid rats are significantly higher and lower, respectively, than those of hepatocytes from euthyroid rats. In a minimal incubation medium (i.e., in one in which the only added substrates are glucose, lactate, and pyruvate), we have found that hyperthyroid rates are 65% greater than euthyroid rates and that hypothyroid rates are 13% lower than euthyroid control rates (15).

We have applied top-down elasticity analysis in hepatocytes to determine whether similar results would be obtained in the intact cells as in liver mitochondria. Our purpose was therefore to quantitatively identify the significant subcellular sites of action of thyroid hormones that are responsible for these changes in cellular oxygen consumption. In hepatocytes from hyperthyroid and euthyroid control rats the analysis of the kinetics of the proton leak, phosphorylation, and substrate oxidation blocks of reactions to Ψ_m showed that the rates of the proton leak and the phosphorylation blocks of reactions were greater in the hyperthyroid hepatocytes (16). These results are shown in Figure 3. The curves representing the kinetics of the proton leak (Figure 3A) show that at any given Ψ_m in hyperthyroid hepatocytes the rate of the proton leak is at least 2.5-fold greater than that in euthyroid hepatocytes. This finding is consistent with the results of Hafner *et al.* (11) in their study of liver mitochondria from hypothyroid, euthyroid, and hyperthyroid rats. There were no differences in the kinetics of the substrate oxidation block of reactions to Ψ_m between hyperthyroid and euthyroid hepatocytes (Figure 3B).

In contrast to our earlier finding of no change in the phosphorylation reactions in liver mitochondria from hyperthyroid rats (15), the results from hepatocytes (16) shown in Figure 3C show clearly that the activity of the phosphorylation reactions is greater in hyperthyroid hepatocytes than in euthyroid hepatocytes. As the phosphorylation block of reactions in liver mitochondria, unlike that in intact cells, does not include the activity of the extramitochondrial ATPases, these findings suggest that the extramito-

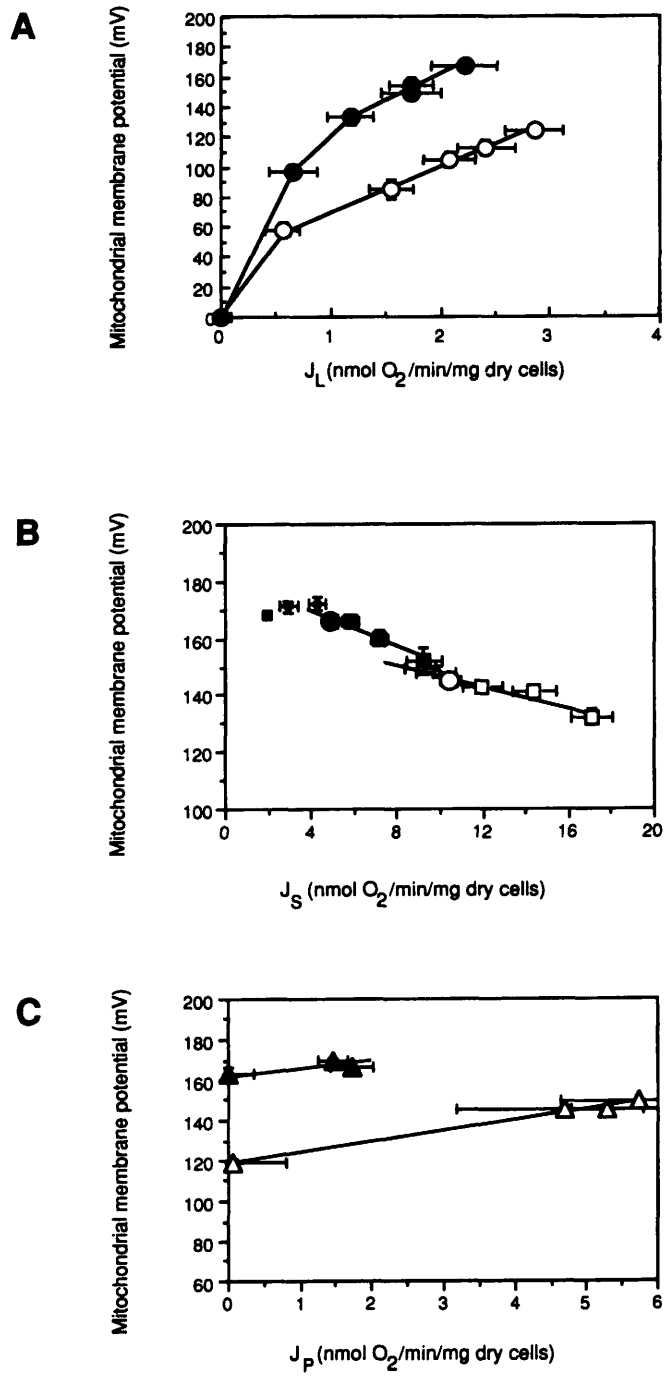

Figure 3. Comparison of the overall kinetic responses of the proton leak, substrate oxidation, and phosphorylation blocks of reactions to $\Delta\Psi_m$ in hyperthyroid hepatocytes and euthyroid control hepatocytes. Oxygen consumption rates are corrected for nonmitochondrial oxygen consumption. Open symbols: hyperthyroid hepatocytes; filled symbols: euthyroid hepatocytes. The large circles (in B) represent the resting points (i.e., in the absence of FCCP and oligomycin). (A) The kinetic response of the mitochondrial proton leak to $\Delta\Psi_m$ (myxothiazol titration of oligomycin-inhibited respiration), showing that the hyperthyroid state results in an increased rate of proton leak at any given $\Delta\Psi_m$. (B) The kinetic response of the substrate oxidation subsystem to $\Delta\Psi_m$. Lines were fitted by linear regression to the FCCP titration points (1.0, 2.0, and 4.0 μM indicated by squares) and oligomycin titration points (0.05, 0.1, and 0.5 μg/ml; indicated by small circles) which delineate the kinetics of the substrate oxidation subsystem. However, as $\Delta\Psi_m$ began to drop in euthyroid cells when the two highest concentrations of oligomycin were used, indicating some secondary effects of high concentrations of oligomycin, these data points (furthest to the left in the plot) were not included when the line was fitted. Results show that hyperthyroidism produces no difference in the kinetics ($P > 0.05$ by analysis of covariance) of the substrate oxidation subsystem. (C) The kinetic response of the phosphorylation subsystem to $\Delta\Psi_m$. This was a myxothiazol titration (0.05, 0.1, and 0.2 μM) of the resting respiration rate minus the rate of oxygen consumption used to balance the proton leak. Results show that hyperthyroidism causes a significant difference in the kinetics of the phosphorylation subsystem to $\Delta\Psi_m$ ($P < 0.05$ by analysis of covariance). Each point represents a mean ± SEM. Results are from cell preparations from the following numbers of hyperthyroid and littermate-paired euthyroid rats: 12 (A), 7 (B), and 3 (C). Each $\Delta\Psi_m$ determination was carried out in triplicate; each oxygen consumption determination was carried out simultaneously in duplicate.

chondrial ATP-consuming reactions are the important targets of thyroid hormones in the phosphorylation block of reactions. The findings support the findings from studies of individual components of the phosphorylation reactions that thyroid hormones affect the activity of the extramitochondrial ATP-consumers, Na,K-ATPase, Ca-ATPase and gluconeogenesis (32–33) but do not support the theory that thyroid hormones have a significant effect on respiration via effects on the kinetics of the adenine nucleotide translocator (25, 27–30) and in the activity of the ATP synthase (24, 31).

The results showing that the overall kinetics of the substrate oxidation block of reactions are unchanged in hyperthyroid hepatocytes compared with euthyroid hepatocytes (Figure 3B [results taken from Ref. 16]) agree with previous results from isolated mitochondria (at intermediate rates of respiration) (13, 15) and with hypothyroid hepatocytes (14), and indicate that any changes in the concentration or activity of the dehydrogenases and respiratory chain components result in insignificant changes in the response of the substrate oxidation subsystem to Ψ_m under the incubation conditions and cannot cause the observed differences in oxygen consumption rate between hyperthyroid and euthyroid hepatocytes.

As described by Brand (10), the overall titration curves that are used to construct the curves representing the kinetics of the three blocks of reactions (Figure 3) can be used to determine the proportions of cellular oxygen consumption that are used in the resting state for phosphorylation and leak blocks of reactions and for nonmitochondrial oxygen consumption (Figure 4). Nonmitochondrial oxygen consumption was identified as that which is insensitive to saturating amounts of oligomycin and myxothiazol (indicated by the shaded bar in each graph in Figure 4 [values are taken from Ref. 16]). Proton leak-dependent oxygen consumption in the resting state was measured as the rate of the proton leak (oxygen consumption in the presence of

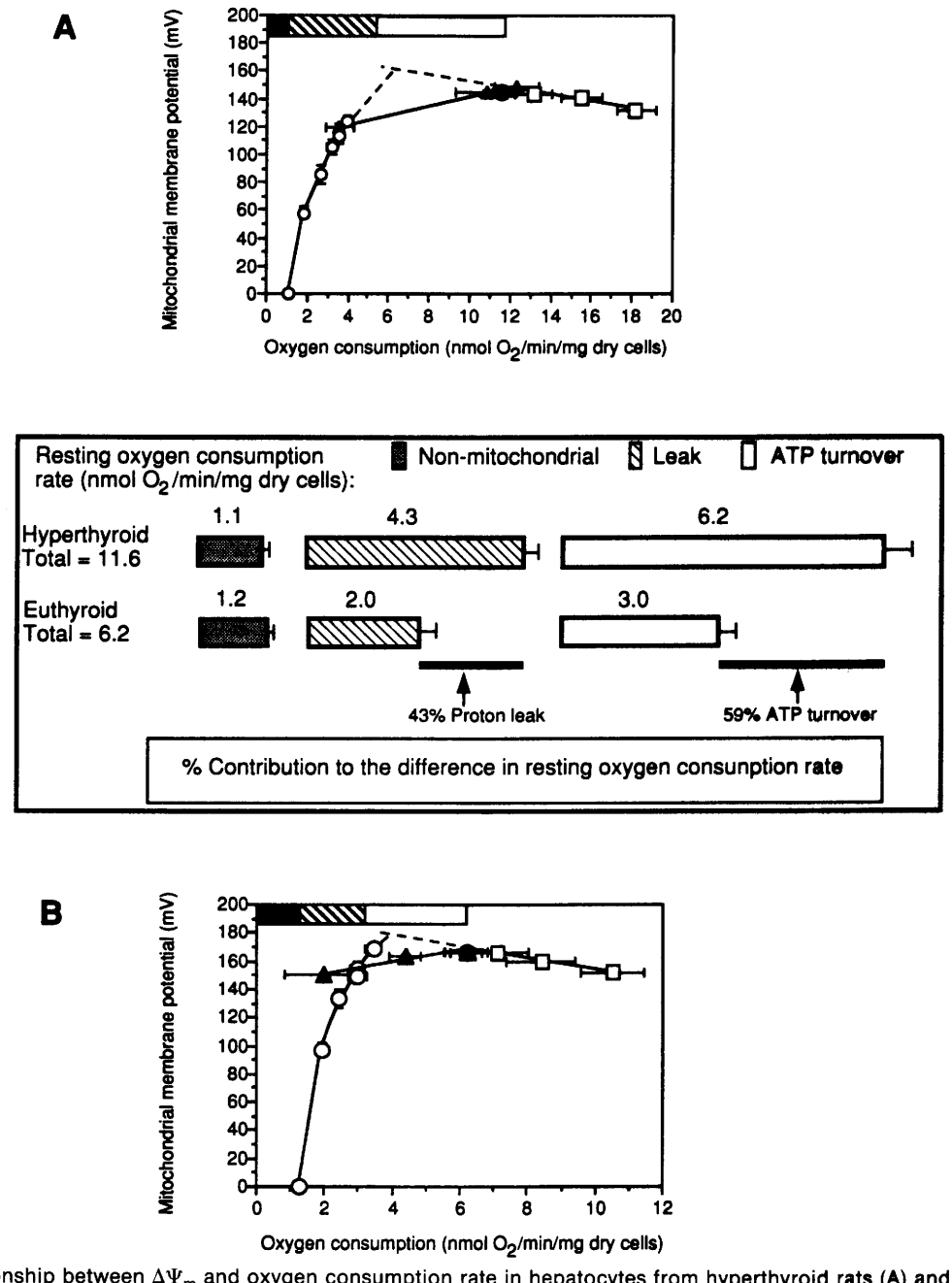

Figure 4. Relationship between $\Delta\Psi_m$ and oxygen consumption rate in hepatocytes from hyperthyroid rats (A) and littermate-paired euthyroid controls (B). Titrations were carried out from the resting cellular respiration state (intersection point of the linear titration curves). The overall kinetic response of the substrate oxidation reactions to $\Delta\Psi_m$ (□) was measured by titrating the $\Delta\Psi_m$ consumers with FCCP (1.0, 2.0, and 4.0 μM). The kinetic response of the proton leak (○) to $\Delta\Psi_m$ was measured by titrating the $\Delta\Psi_m$ producers with myxothiazol (0.05, 0.10, 0.20, and 0.50 μM) in the presence of saturating concentrations of oligomycin (1.0 μg/ml). To estimate the State 4 point the curve representing the kinetics of the leak was extrapolated by eye to the point of intersection with the line representing the kinetics of the substrate oxidation subsystem. (Dashed lines represent the sections of the lines which were determined by extrapolation). The combined elasticity of the $\Delta\Psi_m$ consumers (ATP synthesis and consumption, plus proton leak) (△) to $\Delta\Psi_m$ was measured from titrations with myxothiazol alone (0.05, 0.10, and 0.20 μM). Results are from cell preparations from the following numbers of hyperthyroid and littermate-paired euthyroid rats: 12 (○), 7 (□) and 3 (△). Each $\Delta\Psi_m$ determination was carried out in triplicate; each oxygen consumption determination was carried out simultaneously in duplicate.

The bar graphs in the top left corner of A and B indicate the proportions of oxygen consumption that were due to nonmitochondrial, proton leak, and phosphorylation reactions. The central panel compares these bar graphs and indicates how the difference in resting respiration rate between the hyperthyroid and euthyroid states is distributed. The SEM (for nonmitochondrial oxygen consumption) and pooled SEM (for proton leak-dependent and ATP turnover-dependent oxygen consumption) are indicated; the SEM for the point on each proton leak curve at the resting $\Delta\Psi_m$ was estimated as the mean of the SEM for the two data points adjacent to it.

oligomycin, titrated with myxothiazol) at the same value of Ψ_m as in resting cells (hatched bars in Figure 4). The oxygen consumption that remains is used to support the phosphorylation reactions (clear bars in Figure 4). The diagram between the titration curves compares these bar graphs and shows how the difference in oxygen consumption between the hyperthyroid and euthyroid states is distributed. Of the difference in resting oxygen consumption rates between hyperthyroid and euthyroid hepatocytes, approximately 43% could be accounted for by differences in the rate of the proton leak. There were no significant differences in nonmitochondrial oxygen consumption. The remainder of the difference in resting oxygen consumption rate between hyperthyroid and euthyroid hepatocytes (59%) was due to a difference in ATP turnover-dependent oxygen consumption.

Finally, when the same approach was used to determine the quantitative effects of the changes in the kinetics of the blocks of reactions in the oxidative phosphorylation system in hepatocytes from hypothyroid rats (14), it was found that a decreased rate of the proton leak accounted for 52% of the difference in resting oxygen consumption between hypothyroid and euthyroid hepatocytes. There was no significant difference in the rate of ATP turnover-dependent oxygen consumption between hypothyroid and euthyroid cells. (The rate of ATP turnover-dependent oxygen consumption was slightly higher in hypothyroid cells such that it accounted for approximately 4% of the difference in resting oxygen consumption.) The balance of the difference was attributed to a decrease in nonmitochondrial oxygen consumption which most likely was due to altered microsomal and/or peroxisomal oxidative reactions, considering the results of Ram and Waxman (39) who showed that hypothyroidism in rats resulted in a 75%–85% decrease in hepatic microsomal P450 reductase activity and protein.

Conclusion

The ease with which the top-down approach can be applied, as well as its wide applicability to metabolic systems, makes it an ideal tool for the identification of the sites of regulation by hormones and other external effectors within metabolic systems. Moreover, it provides a means for the determination of the quantitative importance of changes in reactions, or blocks of reactions, towards overall changes in the flux through the system being studied. Because top-down elasticity analyses provide all of the data that are needed for the completion of a metabolic control analysis of the system, one can determine whether the distribution of control within the system is changed with the addition (or removal) of the hormone. The approach has provided a substantial amount of quantitative information on the sites of action of thyroid hormones on oxidative phosphorylation in rat liver mitochondria and hepatocytes. Results from intact hepatocytes have indicated the importance of the mitochondrial proton leak in hypothyroid- and hyperthyroid-induced changes in resting cellular oxygen consumption. Approximately 50% of the changes in resting oxygen consumption in hypothyroid and hyperthyroid states is due to decreased and increased rates of leak, respectively. Results have also shown that changes in nonmitochondrial oxygen consumption in hypothyroid cells and changes in ATP turnover-dependent oxygen consumption in hyperthyroid cells account for the remaining 50% of the changes in oxygen consumption in hypothyroid and hyperthyroid cells.

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