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# KINETIC AND PHYSICO-CHEMICAL ANALYSIS OF ENZYME COMPLEXES AND THEIR POSSIBLE ROLE IN THE CONTROL OF METABOLISM

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## I. INTRODUCTION

Although about 30 years ago Siekievitz (1959) already speculated about the possible role of reversible binding of "soluble" enzymes to cellular membranes in regulation of enzymatic activity, Green *et al.* (1965) were the pioneers suggesting that membrane fractions were capable of catalysing the complete sequence of glycolysis and all metabolic sequences might be membrane bound *in vivo*.

Indeed, the homogeneous components of the cell, like enzymes and metabolites, are immersed into a radically inhomogeneous environment so that "the dynamics of the homogeneous components must need to be coupled into the inhomogeneity of the internal environment" (Elsasser, 1969). In the last two decades more and more authors have suggested that even the so called "soluble" proteins of the cytoplasm and mitochondria may associate with each other or with various membranous and particulate components of the

cell (Atkinson, 1969; Ling, 1969; Kempner and Miller, 1968; Sols and Marco, 1970; Masters, 1981; Srere, 1982, 1987; Friedrich, 1984; Welch, 1977a, 1985; Keleti *et al.*, 1977; Welch and Clegg, 1986; Srivastava and Bernhard, 1986a, 1987a; Damjanovich *et al.*, 1986; Keleti and Ovádi, 1988). Moreover, the intracellular distribution of enzymes may not be an invariant property, but it may vary with the metabolic status of the cell; hence, such terms as "mitochondrial matrix" enzymes, "cytoplasmic" enzymes, etc., may not be correct in their original meaning. The rapid and reversible variation in "localization" may represent one mechanism of several by which regulation of catalytic activity may be achieved (Wilson, 1978; Masters *et al.*, 1987; Ovádi, 1988).

However, a number of controversial data have been accumulated over the past 15 years. Any unequivocal demonstration of an interaction between components of cytosol and membranous structures is fraught with considerable technical difficulties, which relate to the possible perturbations of cellular structure during homogenization, the common use of non-physiological suspension media and the susceptibility of the cytosol to component redistribution during subcellular fractionation (Masters, 1981). Hence, even if there is a quasi-permanent organizational network, stabilized perhaps by weak bonding to membrane or other structures or to other enzymes, the shearing forces of cell disruption can reduce the viscosity sufficiently for it to be destroyed (Ottaway and Mowbray, 1977).

The apparent concentration of several enzymes in the cell is $10^{-6}$–$10^{-5}$ mol/kg of tissue or even higher (Srere, 1967; Bernhard, 1988). These concentrations are much higher than those used generally in kinetic studies *in vitro*, so that the classic kinetic parameters, namely the Michaelis constant and maximum velocity may be significantly altered in the cellular milieu. Moreover, the binding of enzymes to cell particles or other enzymes may also alter both of these kinetic characteristics. Regulatory behaviour may appear or disappear in soluble form or at assay concentration under *in vitro* conditions.

Another thing that also should be kept in mind is the following: tissues in animals contain about 1000 different enzymes of an average molecular mass of $10^5$ (Srere, 1967) and the mean distance between enzymes is well below the diameter of an average tetrameric protein molecule (Srere, 1982), i.e. enzymes are densely packed in the cell and cellular particles. This dense packing would make possible metabolic interactions or regulation controlled simply by diffusion rather than specifically organized structures. Really, if the medium is water, enzymic reactions do not require special conditions or organized structures to allow enzymes to encounter their substrates and effectors: that is, diffusion may be rapid enough that it could not be the rate limiting factor (Webb, 1963). However, in an intracellular "microenvironment" diffusion could be rate limiting in the enzymic reaction (Hübscher *et al.*, 1971).

The extremely high macromolecular crowding of the cytoplasm as well as that of the mitochondria influences the biorecognition processes through alteration of the kinetics and equilibria of reactions leading to the formation of specific macromolecular complexes, such as the binding of hormones to their receptors, the recognition of foreign substances by antibodies, etc. (Minton, 1987). Recently, new techniques have being developed for quantification of macromolecular association equilibria at concentrations far higher than previously possible and also in the presence of other species of macromolecules at concentrations comparable with the physiological ones (Chatelier and Minton, 1987a,b). Model calculations to high background protein concentrations suggest that in solutions containing proteins at concentrations comparable to those existing in biological media, the diffusive transport of larger proteins and aggregates is slower than in dilute solution by several orders of magnitude (Muramatsu and Minton, 1988), while the diffusion of small molecular mass compounds (like metabolites, effectors) slowed down 2–3 times only (Mastro *et al.*, 1984; Jacobson and Wojcieszyn, 1984). This was explained as the result of mostly transient interactions of the diffusible macromolecules with the cytoplasmic matrix proteins (Gerson *et al.*, 1985). Taking into account the sufficiently viscous cytoplasm and membranes, the appropriate metabolite concentrations can be created by arranging enzymes of a certain pathway next to each other.

A number of instances are known where enzymes remain attached to each other during extraction and purification procedures. Such physically associated multienzyme systems,

independently of the nature of their physical association, have the potential of exhibiting unique catalytic properties (Gaertner, 1978). A multienzyme complex is defined as an aggregate of different, functionally related enzymes bound together by non-covalent forces into a highly organized structure (Ginsburg and Stadtman, 1970). Multienzyme complexes do not seem to be random associations of enzymes but ones which are related by virtue of catalysing sequential reactions and they allow channelling of metabolites along specific enzyme pathways without the intermediates becoming free (Stebbing, 1980). However, the same enzymes in closely related organisms may be differently associated and we should therefore be wary of applying one explanation for the existence of multienzyme complexes in general. Multienzyme complexes are likely to reveal functions and origins in intermediary metabolism. However, enzyme–enzyme interactions occur not only between components of well established multienzyme complexes, but also between so-called "soluble" enzymes. Although these interactions are weak and the enzymes can be extracted from the cell as individual entities, they may ensure a dynamism for the complexes which manifests itself in their dissociation–association processes (Jaenicke and Helmreich, 1972; Salerno *et al.*, 1975). Such interactions are assumed to have functions *in vivo* and this point applies especially to areas of current interest: compartmentation or channelling, understanding of which phenomena might provide explanation of metabolic controls. Indeed, development of the concept of metabolic compartmentation seemed to be necessary since several data could not be understood in any other way (Srere and Mosbach, 1974; Ovádi and Keleti, 1978; Friedrich, 1984; Keleti and Ovádi, 1988).

## II. METHODS FOR QUALITATIVE AND QUANTITATIVE ANALYSIS OF MACROMOLECULAR INTERACTIONS

### 1. *Physico-chemical Methods*

Since the interactions are frequently loose and transient in dynamic enzyme complexes, their study is difficult. Special sensitive physico-chemical techniques are required to detect complex formation of enzymes as well as to characterize quantitatively the heterologous interactions. From these data the possible existence of enzyme complexes at physiological enzyme concentrations could be deduced.

#### (a) *Sedimentation methods (velocity and equilibrium sedimentation, active band ultracentrifugation)*

The sedimentation of an enzyme complex existing in equilibrium with their components may be characterized by its mass average sedimentation coefficient, $s$, which is the average of the sedimentation coefficients of all components in terms of their mass concentrations in solution. For a monomer–dimer system Gilbert (1955) has shown that a single sedimenting boundary is observed at all concentrations if the rate of association is very rapid compared to that of dissociation; however, in such a system $s$ shows a characteristic concentration dependence.

With the development of the different sedimentation technique (Kirschner and Schachman, 1971), which has the precision to detect extremely small alterations in sedimentation coefficient, the problem of distinguishing very small changes in molecular mass from changes in frictional coefficient has largely been overcome.

Measurements over a range of protein concentrations do justify conclusions about the magnitude of the association constant (Smith *et al.*, 1973; Zimmerman and Crowl-Powers, 1988). Ligand-induced changes in association constant would be recognized and the difference in sedimentation coefficient could be attributed to a shift in the equilibrium.

The active enzyme ultracentrifugation method allows one to obtain the hydrodynamic parameters of the enzyme–substrate complex while it is fully active. The sedimentation and diffusion coefficients of the enzyme–substrate complex are calculated from the optical observation of either the appearance of the product of the reaction or the disappearance of the substrate (Cohen and Mire, 1971). Although this method may produce artifacts, not found in the more common centrifugation methods, it has numerous advantages. One of

them is, for example, that the sedimentation of the enzyme can be observed at very dilute concentrations, hence the hydrodynamic properties can be directly compared with kinetic data. Moreover, this method does not require a purified preparation; therefore, it may be advantageous to investigate enzymes associated in complexes.

During the ultracentrifugation run of two proteins the symmetrical difference curve of two successive absorption scannings may become asymmetric. According to the consideration of Cohen and Claverie (1975) such a shoulder is a direct proof of interaction between active species having different molecular mass. Consequently, it was suggested that the asymmetrical shape of the difference curve is indicative of complex formation between two enzymes. It should be mentioned that in such a complex case the quantitative interpretation is rather difficult, if not impossible (Llewellyn and Smith, 1978).

Until now it was not completely clear why complex formation between soluble globular enzymes detected kinetically in some cases could not be demonstrated by ultracentrifugation methods (Horecker *et al.*, 1981; Fahien and Smith, 1974) with an exception of the complex between aldolase and glycerol-3-phosphate dehydrogenase, as indicated by active band ultracentrifugation (Batke *et al.*, 1980). The reason for the failure to demonstrate complex formation is probably connected with the pressure effect known for many years and discussed in detail by Harrington and Kegeles (1973). The high pressure ($7 \times 10^6$–$40 \times 10^6$ Pa) in the ultracentrifuge cell during a run can change the molecular volume and shift dramatically the equilibrium of the complex. This effect may be especially significant in high speed velocity experiments, which, otherwise, would be advantageous for the analysis of "labile" systems, since a relatively short time is needed for the run compared to the sedimentation equilibrium studies, which requires slower rotor speed (consequently pressure effect becomes less important). If the high pressure induces the decomposition of enzyme complexes, the detection of such an enzyme complex may be possible by active band ultracentrifugation applying the non-functioning enzyme in high excess relative to the "active" enzyme, the hydrodynamic parameters of which are monitored. Binding of soluble proteins to the skeletal elements of the cell has been detected by ultracentrifugation techniques (*cf.* Section III.3.b).

(b) *Chromatographic methods (gel-, affinity-chromatography, immobilization)*

Gel-chromatographic methods are utilized for determination of stoichiometry and equilibrium constants of associating protein systems. The method is very commonly used since it requires simple equipment and it is particularly well-suited to studies in a wide range of concentrations. The basic principles of gel-chromatography and applications to single and polydisperse solute systems have been described in several reviews (Ackers, 1970, 1975). The technique of elution and batch chromatography is based upon the molecular size-dependent penetration of solute molecules into porous networks of gels such as crosslinked dextrans (Sephadex), polyacrylamide and agarose. This technique has been used, for example, to detect complex formation between aldolase and glycerol-3-phosphate dehydrogenase and to study the modulating effect of metabolites on this heterologous enzyme interaction (Vértessy and Ovádi, 1989).

Small zone elution gel-chromatography is a useful qualitative method of detecting interaction, but from such an experiment association constants cannot be determined.

In large zone experiments in the case of dissociable systems the data should be derived from a wide range of concentrations in order to carry out an unequivocal determination of stoichiometry and provide some diagnostic information as to the type of system (Valdes and Ackers, 1979).

The fundamental principle of affinity chromatography is the utilization of the exceptional property of biologically active substances to form stable specific and reversible complexes. If one of the components of the complex is immobilized, a specific sorbent is formed for the second component of the complex, with the assumption, of course, that all conditions necessary for the formation of this complex are maintained. The binding sites of the immobilized substances must retain good steric accessibility even after their binding to the solid carrier, and they must not be deformed. The application of affinity chromatography to

the study of interactions of enzymes seems very promising, although the results do not permit general conclusions to be drawn (Antonini *et al.*, 1975; Powell and Morrison, 1979). Moreover, these methods are time consuming and the working formulae are rather complicated (Nichol *et al.*, 1974b; Danner *et al.*, 1979), since in affinity chromatography a heterologous system is operative. Nevertheless, this method was employed to demonstrate the substrate-induced dissociation of the tetrameric glyceraldehyde-3-phosphate dehydrogenase (Kálmán *et al.*, 1980). The dissociation constant of the complex between aldolase and glyceraldehyde-3-phosphate dehydrogenase has been determined by using NAD-Sepharose 4B as affinity gel in an equilibrium batch system (Kálmán and Boross, 1982). With the help of this method it is possible to study any kind of interaction between two molecules if there exists any affinity sorbent for one of the interacting molecules, while the other molecule cannot bind to this sorbent. Furthermore, this system is easily applicable to the study of factors influencing complex formation, e.g. pH, temperature and ionic strength.

The immobilization of enzymes is important from both theoretical and practical points of view. Enzymes bound to well characterized surfaces of solid supports represent simple models for the study of the effect of microenvironments on the binding and transformation of substrate. As most enzymes *in vivo* are bound to membranes or occur in the form of some other complex of the native environment, the study of such systems is undoubtedly important. Many reviews have already been written on affinity chromatography and the binding of enzymes to solid supports (Cuatrecasas, 1972; Cuatrecasas and Anfinsen, 1971a, b; Mosbach, 1977; Turkova, 1978).

Immobilization has been used to detect subunit–subunit interactions in oligomeric enzymes (Nagradova *et al.*, 1981; Muronetz *et al.*, 1982, 1986; Ashmarina *et al.*, 1980, 1982) or heterologous complex formation between two different enzymes (Ashmarina *et al.*, 1984, 1985, Muronetz *et al.*, 1986; Arrio-Dupont *et al.*, 1985).

Immobilization of two sequentially working enzymes on the same matrix was described by Mosbach and coworkers (Mosbach and Mattiasson, 1970; Siegbahn *et al.*, 1987). The rate of the overall reaction catalysed by the enzymes coupled together on a carrier was compared with the rate of the corresponding free enzymes equivalent in activity to those of the immobilized ones. It was concluded that in an immobilized system the product of the reaction catalysed by the first enzyme is available in a higher concentration in the environment of the second enzyme, than in the system of free enzymes. This two-enzyme system was then extended to a three- (Srere *et al.*, 1973; Mattiasson and Mosbach, 1971) and four-enzyme system (Okamoto *et al.*, 1980). Moreover, the enzymes of a complete metabolic cycle, the urea cycle, have been co-immobilized to supports (Siegbahn and Mosbach, 1982). In all cases the immobilized systems were much more efficient than the corresponding soluble ones.

Mansson *et al.* (1983) described the use of bis-NAD analogues to obtain an immobilized two-enzyme system (lactic dehydrogenase and alcohol dehydrogenase) in which the two different active sites are facing one another. Similar experiments were performed with an oxidase/peroxidase system using quinones as crosslinking intermediates (Dittrich and Neumann, 1988). By such an arrangement the diffusion of the product of the first enzyme to the active site of the second enzyme was facilitated due to the proximity and proper orientation of the active sites.

(c) *Partition (in polyethylene glycol) method*

Precipitation by polyethylene glycol (PEG) is a simple and suitable method to detect an association–dissociation process in enzyme systems where either homologous or heterologous interaction occurs.

Ogston (1937) argued many years ago that PEG provided an environment for proteins more comparable to the cellular environment, where they are surrounded by a high concentration of hydrophobic protein molecules, than do most aqueous solutions. This insight is probably more valid for the highly-concentrated proteins of the mitochondrial matrix than it is for proteins in other subcellular compartments, but even in those other compartments protein concentrations may reach 20%, a concentration seldom used in the

study of proteins *in vitro*. The use of PEG as a "crowding agent" in the study of the kinetic behaviour of mammalian phosphofructokinase opened ways for the investigation of enzymes at physiological concentrations (Boscá *et al.*, 1985). This approach was used to detect the interaction of phosphofructokinase and fructose-bisphosphatase (Ovádi *et al.*, 1986).

PEG is one of the most useful protein salting-out agents. It has been shown that the salting-out effectiveness of PEG can be explained by the large unfavourable change in free-energy of its interaction with proteins (Arakawa and Timasheff, 1985). Results from solvent–protein interaction studies indicate that PEGs are hydrophobic in nature and will interact favourably with the hydrophobic side chains exposed upon unfolding (Lee and Lee, 1987).

Coprecipitation of two enzymes in the presence of PEG under conditions when neither alone is precipitated could be due to the interaction between them; however, the molecular basis of the protein-precipitating action of PEG and other polymers is poorly understood. The effectiveness of PEG increases with the size of the polymer (Polson *et al.*, 1964) and the larger proteins tend to precipitate at lower concentrations of PEG (Juckes, 1971). Hence, it seems probable that the proteins are sterically excluded from regions of the solvent occupied by the inert synthetic polymers and are thus concentrated until their solubility is exceeded and precipitation occurs. For example, interaction of glutamate dehydrogenase with mitochondrial enzymes and the interaction of citrate synthase with malate dehydrogenase has been demonstrated in PEG (Halper and Srere, 1977).

PEG is one of the polymer components in biphasic systems which is more and more widely used to study interactions between enzymes, and enzymes and particles. If the distribution of two substances in a biphasic system is different from when they are alone, interaction can be detected, and the change in partition can be used for calculation of dissociation constants. The properties of polymers of biphasic systems and the application of the distribution method have been described in detail by Albertsson (1971) and Albertsson *et al.* (1982). The systems are obtained by dissolving two water soluble polymers above certain concentrations in water. Salts and buffers can be added to give a desired ionic strength and pH. The most commonly used system contains dextran and PEG.

The aqueous polymer solutions are not adverse towards biological material and can dissolve many proteins and other biopolymers. A number of macromolecular systems have been studied by this partition technique and interactions could be detected, e.g. protein–protein, protein–nucleic acid complex formation, the binding of proteins to membrane surfaces, cell organelles, membrane vesicles. It should be stressed that the dissociation constants obtained by this method using such a milieu are not necessarily the same as obtained with water as solvent. The separation of two components can be improved considerably by repeating the partition procedure several times, for example, by the procedure of countercurrent distribution (CCD). By comparison of the CCD diagram for two molecules when they are run separately or together one can draw conclusions as to their interaction. By this technique interaction has been detected between cytoplasmic forms of malate dehydrogenase and aspartate aminotransferase and also between the mitochondrial forms of the two enzymes. However, no interaction was found between the heterotopical enzymes (Backman and Johansson, 1976). Association of glycolytic enzymes with filamentous actin was also detected by CCD (Westrin and Backman, 1983), as well as protein–protein association of six enzymes of the Calvin cycle (Persson and Johansson, 1989). Complex formation of five enzymes of the Calvin cycle was demonstrated also with other independent methods (Gontero *et al.*, 1988).

However, caution is needed in using different synthetic polymers. Several "inert" synthetic polymers (polyvinyl alcohol, polyvinylpyrrolidon) inhibit or activate different enzymes (aldolase, glyceraldehyde-3-phosphate dehydrogenase) by direct interaction and even change their mechanism of action (Jancsik *et al.*, 1976, 1979; Keleti *et al.*, 1977, 1978).

(d) *Fluorescence methods (steady-state intensity and anisotropy measurements, energy transfer, time-resolved anisotropy techniques)*

The study of interacting macromolecular systems by fluorescence techniques is a

productive, thriving endeavour. Changes in fluorescence intensity (quantum yield) and in the fluorescence polarization can be considered as sensitive indicators of environmental effects which alter both the conformation of proteins (static and dynamic quenching, *cf.* Eftink and Ghiron, 1981) and/or the aggregational state of oligomers or heterologous enzyme–enzyme complexes (*cf.* Hammes, 1981; Rawitch and Weber, 1972).

In practice signals originating from intrinsic groups of the proteins (tyrosine, tryptophan and prosthetic groups such as flavine and pyridoxal cofactors, etc.) and externally attached fluorescence dyes (probes) are used for detection. Increase in the size of a macromolecule due to the formation of a complex with another macromolecule can be followed by the change of the fluorescence quantum yield ($y$) or intensity ($I$) and/or fluorescence polarization ($p$) or rather the steady state emission anisotropy ($r$). The advantage of using anisotropy in calculations is that anisotropies are additive whereas summation of polarization is a complex procedure (Jablonski, 1960; Deranleau *et al.*, 1980). The steady state anisotropy ($r$) is:

$$r = 2p/(3-p) \quad \text{and} \quad p = (I_{vv} - I_{vh}G)/(I_{vv} + I_{vh}G)$$

where $G = I_{vh}/I_{hh}$ and $I$ is the fluorescence intensity of the emitted light. The first and the second indices refer to the positions ($v$: vertical, $h$: horizontal) of the polarizer and analyser, respectively. $G$ is the instrumental factor. The additivity of fluorescence emission anisotropies can be represented by

$$r = \sum_i f_i r_i$$

where $r$ is the measured anisotropy of fluorescence emitted by a mixture of molecular species excited at a given wavelength by linearly polarized light, $r_i$ is the anisotropy due to species $i$ and $f_i$ is the fraction of the total emitted fluorescence of species $i$.

In fluorescence measurements special care is needed to avoid artifacts caused by concentration quenching and known as inner-filter effects. A correction formula for the calculation of quenching both in the excitation and emission processes has been derived and applied for quantitative analysis of the concentration-dependent dissociation of the tetrameric glyceraldehyde-3-phosphate dehydrogenase into dimers and monomers (Batke, 1982). The values of the dissociation constants calculated with this method agreed well with those obtained from fluorescence anisotropy measurements using the formula originally proposed by Rawitch and Weber (1972) and extended by Ovádi *et al.* (1982) for the analysis of two-step dissociation processes.

A large variety of information can be obtained from singlet–singlet fluorescence energy transfer measurements. This method was elaborated by Förster (1951, 1965) and others (Jovin, 1979; Stryer, 1978) for the determination of distances in macromolecules in the range of 100–800 nm; however, it is also elegantly suited to the study of stoichiometry, conformational changes and kinetics of complex formation in associating protein systems. In double sphere transfer experiments one of the components of the complexes was labelled only with donor chromophores and the other only with acceptor chromophores and the transfer was indicated in binary and also in ternary complexes of trypsin, alpha-chymotrypsin, and the double-headed black-eyed pea inhibitor (Gennis *et al.*, 1972).

In fact, the double sphere transfer method may be useful for *in vivo* demonstration of enzyme complex formation in intact cells or in cell suspensions by measuring the transfer efficiency between the separately (covalently) labelled purified components after injecting them into the cell or mixed to the cell-suspension.

Techniques permitting the semiquantitative interpretation of singlet–singlet energy transfer measurements on multiply labelled single proteins and protein complexes are given by Gennis and Cantor (1972). In a complex of two or more proteins singlet energy transfer measurements enable one to determine fairly accurately the distances between pairs of proteins (Gennis and Cantor, 1972; Laskowski and Sealock, 1971).

Time dependent anisotropy, $r(t)$ (*cf.* Tao, 1969) is also used in the study of interacting enzymes and proteins (Churchich and Lee, 1976; Ikkai *et al.*, 1980; Kim *et al.*, 1988). The complexes of aspartate aminotransferase/glutamate dehydrogenase and aspartate amino-

transferase/pyridoxal kinase were characterized by larger rotational correlation times, 175 and 62 nsec, respectively, than the free aspartate aminotransferase for which values of 43 nsec (Churchich and Lee, 1976) and 36 nsec (Kim *et al.*, 1988) were reported.

The physico-chemical detection of the formation of multienzyme complexes is the *conditio sine qua non* to start experiments concerning their role in the regulation of metabolism. However, complex formation without kinetic consequences is irrelevant as far as direct regulation is concerned.

## 2. *Kinetic Methods*

While the functional advantages of the stable multienzyme complexes in the regulation of cellular metabolism have been extensively investigated, and are widely acknowledged, the dynamic enzyme complexes being loose and transient in nature, are much less well characterized. The kinetic approaches are the widely accepted techniques to study the functional consequences of enzyme interactions. The kinetic method for the detection and analysis of enzyme complexes is based upon measuring the steady-state flux in consecutive reactions catalysed by functionally related enzymes as well as determining the time required for attaining the steady-state flux. Modelling of the interacting enzyme systems makes it possible to quantify the heterologous enzyme interactions.

The possible theoretical approaches of the kinetic analysis of the *in vitro* interaction of two functionally related enzymes are the following: (i) the analysis of deviation from the kinetics of coupled enzymes, assuming no interaction between the enzymes; and then (ii) the analysis of the kinetics of interacting coupled enzymes by developing different mathematical models.

### (a) *Transient time and steady-state velocity of coupled reactions*

If the reaction catalysed by the first enzyme proceeds at constant velocity, and the reaction catalysed by the second enzyme is first order in respect of the intermediate (which is the product of the first and the substrate of the second enzyme) then the linear part of the time course represents the steady-state velocity of the first enzyme. The production of the end product reaches the steady-state as a function of time after a lag-phase. The intercept of the extrapolated linear part on the time axis equals the transient time, $\tau$, which is proportional to the lag-phase (Hess and Wurster, 1970; Bartha and Keleti, 1979; Keleti, 1984; Easterby, 1981, 1986).

Interaction between the two enzymes can be kinetically analysed by measuring the time courses of the coupled reaction at different enzyme concentrations keeping their ratio constant (Hess and Wurster, 1970). If a kinetically significant complex is formed, then the steady-state velocity will not be a linear function of the concentration of the first enzyme and/or the change in $1/\tau$ as a function of the concentration of the second enzyme will not be linearly proportional.

By increasing the concentration of the first enzyme ($E_1$) the concentration of the intermediate ($I$) is also increased resulting in a hyperbolic dependence of steady-state velocities on $I$ concentrations, from which the $K_m$ value of $I$ for the second enzyme ($E_2$) can be calculated for the coupled system:

$$v = k_{cat}[E_2]_T(k_1[E_1]_T t - [P])/\{K_m + (k_1[E_1]_T t - [P])\} \quad (1)$$

where $k_1$ is the first order rate constant of the reaction catalysed by $E_1$ and $P$ is the end product of the coupled reaction, $K_m$ is the Michaelis constant.

If this $K_m$ value differs from that measured in a separate reaction (when only $E_2$ was present) then a kinetically significant interaction between $E_1$ and $E_2$ exists. Moreover, this phenomenon may be indicative of the existence of direct transfer of the intermediate between the active centres of two enzymes (Ovádi and Keleti, 1978).

One should bear in mind that if the reaction of $E_2$ is not of first order with respect to $I$, but follows Michaelis–Menten kinetics, then (Storer and Cornish-Bowden, 1974):

$$\tau = K_M/(V_{max} - v_1). \quad (2)$$

In a coupled system of two enzyme-catalysed reactions, provided that the activities of the

two enzymes are comparable, the kinetic properties of both enzymes may be determined simultaneously by analysis of the sigmoidal progress curve of product formation (Duggleby, 1983). Kuchel *et al.* (1974) have showed that for a coupled enzymatic sequence of $n$ enzymes, each of which has a single substrate and obeys Michaelis–Menten kinetics, a plot of the concentration of the final product vs $t^n$ will tend towards a straight line. For more general theoretical treatments of the transient time, for example, when both enzymes follow Michaelis–Menten or one of them reversible kinetics or the chain of consecutive reactions comprises three or more enzymes, we refer to the literature (McClure, 1969; Rudolph *et al.*, 1979; Easterby, 1973, 1981; Kuchel and Roberts, 1974; Nichol *et al.*, 1974a; Brooks *et al.*, 1984; Keleti, 1986; Yang and Schulz, 1987; Brooks and Suelter, 1989).

For a two-enzyme coupled reaction (if $[E_2] \ll K_{m,E_2}$, *cf.* Bartha and Keleti, 1979) the transient time is the reciprocal of the kinetic power (Keleti and Vértessy, 1986; Welch *et al.*, 1988): $\tau = 1/k_\Gamma$, where the kinetic power, $k_\Gamma = V_{max}/K_M$, which encompasses all factors which bear upon the conversion of free substrate to free product within the cell (Keleti and Welch, 1984; Keleti, 1988). The transient time can be defined not only for the simplest one-substrate reaction as the reciprocal of the kinetic power but also for two-substrate reactions as well as reactions catalysed by allosteric enzymes.

We define the kinetic power of the enzyme catalysing the reaction of two substrates A and B as:

$$k_{\Gamma,A,B} = V_{max}/[(K_{m,A} + K_{m,B})/2] \tag{3}$$

i.e. the kinetic power is the ratio of maximal velocity and the average Michaelis constant (Keleti, 1989a) and it is the function of the ratios of dissociation and decomposition of the central complex and its formation, which follows from the transformation of eqn (3):

$$1/k_{\Gamma,A,B} = (K_{S,A} + K_{S,B})/2k_2[E]_T + (k_{1,A} + k_{1,B})/2k_{1,A}k_{1,B}[E]_T. \tag{4}$$

If $k_{1,A} = k_{1,B} = k_D$, where $k_D$ is the diffusion rate constant,

$$1/k_{\Gamma,A,B} = (1/k_D[E]_T)[(k_{-1,A} + k_{-1,B})/2k_2 + 1] \tag{5}$$

and if $k_{-1,A} \approx k_{-1,B} \approx k_2$, which is identical with the evolutionary "compromise" condition for a simple enzyme function (Knowles, 1976),

$$k_{\Gamma,A,B} = k_D[E]_T/2. \tag{6}$$

If we assume a single-substrate enzyme to be allosteric and to follow the symmetry model (Monod *et al.*, 1965) we obtain for the kinetic power (Keleti, 1989a):

$$1/k_\Gamma = \left[\sum_{i=1}^{n} (K_R^{i-1} + LK_T^{i-1})/(K_R^i + LK_T^i)V_{max} + n(1/k_1[E]_T)\right] \Big/ n \tag{7}$$

where $K_R$ is the association constant of the $ES$ complex in the $R$ state and $K_T$ is the same in the $T$ state. L is the allosteric equilibrium constant of the step $E_T \Leftrightarrow E_R$, i.e. $L = [E_T]/[E_R]$, where $E_R$ and $E_T$ are the free enzyme in $R$ and $T$ state, respectively.

In the case of the tetrahedral sequential model (Koshland *et al.*, 1966) assuming the subunits to exist in 'a' form in the absence and in 'b' form in the presence of bound substrate, the kinetic power is (Keleti, 1989a):

$$1/k_\Gamma = \left[\sum_{i=1}^{n} 1/K_{a,i}V_{max} + n(1/k_1[E]_T)\right] \Big/ n \tag{8}$$

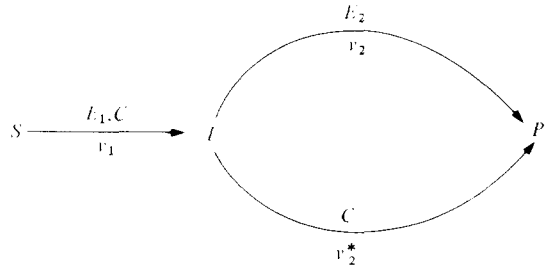
where $K_{a,i}$ are the association constants defined in eqns (9):

$$\begin{aligned} K_{a,1} &= K_b K_c K_d^3 \\ K_{a,2} &= K_b K_c K_d K_e \\ K_{a,3} &= K_b K_c K_e^2 / K_d \\ K_{a,4} &= K_b K_c K_e^3 / K_d^3 \end{aligned} \tag{9}$$

for the four subunits, where $K_b$ is the association constant of $S$ with the b conformation of subunits, $K_c$ is the equilibrium constant of the conversion of the subunit in the a conformation to that in the b one, $K_d$ is the subunit interaction factor between subunits in form a and b, and $K_e$ that between a pair of b subunits. Consequently, the reciprocal of the kinetic power defines the transient time of the coupled reactions even for two-substrate enzymes and for allosteric enzymes.

(b) *Transient time in interacting systems*

Let us analyse quantitatively the following coupled reaction catalysed by two enzymes interacting in a kinetically significant manner (Bartha and Keleti, 1979; Keleti, 1984):




If the first reaction is of zero order, $E_1$ is practically present only in the enzyme–substrate complex ($E_1S$). $E_1S$ forms a complex ($C$) with $E_2$, with the dissociation constant $K$. Both $E_1$ and $C$ transform $S$ into $I$ with the same velocity $v_1$, i.e. it is assumed that $E_2$ does not affect the activity of $E_1$. However, it is assumed that $E_1$ affects $E_2$, i.e. that $E_2$ catalyses the transformation of $I$ into $P$ with the velocity $v_2$, but $C$ catalyses it with $v_2^*$. It is assumed that no channel is formed.

If $n$ is the number of active sites of $E_2$ bound per active site of $E_1$ and the reaction is of first order with respect to $I$:

$$v_2 = (k_2/K_m)([E_2]_T - n[C])[I] \tag{10}$$

$$v_2^* = (k_2^*/K_m^*)n[C][I] \tag{11}$$

where $k_2$, $K_m$ and $k_2^*$, $K_m^*$ are the microscopic rate and Michaelis constants of the reaction of $E_2$ and $C$, respectively.

If $v_1$ = constant and at $t=0$, $[P]=[I]=0$, the law of mass conservation requires that:

$$[P] = v_1 t - [I] - v_2/k_2 - v_2^*/k_2^*. \tag{12}$$

In the steady-state, if $[I]=[I]_{ss}$ = constant, then $[C]=[C]_{ss}$ = constant, $v_1 = v_2 + v_2^*$ = constant, and

$$[P] = v_1(t-\tau). \tag{13}$$

If we assume that $v_1 \ll k_2[E_2]_T$, $k_2^*[E_2]_T$, and $[I]_{ss}$, $[E_2]_T \ll K_M$, $K_M^*$, we obtain

$$v_2 \approx (k_2/K_m)([E_2]_T - n[C]_{ss})[I]_{ss} \tag{14}$$

$$v_2^* \approx (k_2^*/K_m^*)n[C]_{ss}[I]_{ss} \tag{15}$$

$$[C]_{ss} \approx (1/2)([E_1]_T + [E_2]_T/n + K)(1 - \sqrt{\{1 - 4[E_1]_T[E_2]_T/n([E_1]_T + [E_2]_T/n + K)^2\}}) \tag{16}$$

and

$$v_1\tau = [I]_{ss} \tag{16a}$$

$$[E_1]_T/(k_2[E_2]_T/K_m - 1/\tau) + K/(k_2^*[E_2]_T/K_m^* - 1/\tau) \approx (1/n)/(k_2/K_m - k_2^*/K_m^*). \quad (17)$$

Measuring $\tau$ at different $[E_1]_T$ and $[E_2]_T$ one can determine $k_2/K_m$, $k_2^*/K_m^*$ and $K$ by non-linear regression. If $k_2/K_m$ is known from independent measurements and $[E_1]_T = [E_2]_T/n$ then one may estimate $k_2^*/K_m^*$ and $K$ from a linearized form:

$$1/\tau[E_2]_T = k_2^*/K_m^* \pm \sqrt{[K|nk_2/K_m - nk_2^*/K_m^*|]}\sqrt{[(1/[E_2]_T)|k_2/K_m - 1/\tau[E_2]_T|]}. \quad (18)$$

(c) *Analysis of the mechanism of intermediate transfer*

A special consequence of complex formation between two enzymes is the channelling of intermediate, i.e. the direct transport of the product of the first enzyme from its active centre to the active centre of the second enzyme (Nichol *et al.*, 1974a). The channelling of the intermediate substrate may result in physiological advantages to an organized state like (i) segregation of competing pathways due to microcompartmentation of intermediates; (ii) reduction of time required to reach the steady-state; and (iii) enhancement in metabolite flux by providing high local metabolite concentration. The kinetic methods for detection and analysis of channelling are based upon measuring the transient time ($\tau$) in the interacting system (Hess and Wurster, 1970). A new description of the channelling effect on the bases of its inherent parameters such as channel efficiency and intermediate lifetime makes it possible to explain reduction of transient time due to the interaction, even if no changes in the kinetic parameters of the individual reaction occur and no physical barrier prevents the diffusion of $I$ into the bulk solution (Tompa *et al.*, 1987a).

If the enzymes, $E_1$ and $E_2$, catalyse consecutive conversion of the initial substrate ($S$) to the final product ($P$) via formation of intermediate ($I$) then the lifetime of the intermediate includes the times required for release from $E_1$, diffusion time, association to and conversion by $E_2$. The sum of these times for all molecules yields a characteristic lifetime of the whole population (Fig. 1). Obviously, the fraction of molecules ($\alpha$) which will be converted within the $E_1E_2$ enzyme complex ($C$) has a shorter lifetime ($\langle t' \rangle$) than the non-channelled one ($\langle t \rangle$), since the average distance between active centres is shorter within a heterologous enzyme complex than between separated enzyme molecules. The channel efficiency ($\alpha$) can be defined as the probability of an intermediate being converted within the generating complex. If the generation of intermediate proceeds at a constant rate ($v$) and $E_2$ is subsaturated by $I$, then the concentration of $I$ at steady-state ($I_{ss}$) for partially complexed enzyme systems is the following (Tompa *et al.*, 1987a):

$$[I_{ss}] = v/[E_1]_T\{\alpha[C]\langle t' \rangle + ([E_1]_{free} + (1-\alpha)[C])\langle t \rangle\}. \quad (19)$$

Since $[P] = vt - [I_{ss}]$ at steady-state, by extrapolating the linear part of the progress curve of product formation, its intercept on the time axis is the apparent transient time ($\tau_{app}$) which is characteristic for channelling effect in a certain partially complexed enzyme system:

$$\tau_{app} = 1/[E_1]_T\{\alpha[C]\langle t' \rangle + ([E_1]_{free} + \{1-\alpha\}[C])\langle t \rangle\} \quad (20)$$

where $\langle t \rangle$ is a concentration dependent parameter ($K_{m,2}/k_{cat,2}[E_2]$), while $\langle t' \rangle$ is a concentration independent one being an inherent property of the enzyme complex. By measuring $\tau_{app}$ at various enzyme concentrations ensuring different degree of complexation, the channel efficiency and lifetime of the channelled intermediates can be experimentally determined. In general, $\alpha$ varies between 0 and 1. such that

$$\langle t' \rangle \leq \tau_{app} \leq \langle t \rangle.$$

The decrease in transient time in the interacting enzyme system may be due either to the presence of a physical barrier on the out-diffusion of intermediate or to the mere juxtaposition of the sequential active sites (Fig. 1). Any alteration in the interacting system relating either to the diffusional process or to the kinetic parameters, will manifest itself in changes of $\alpha$, $\langle t' \rangle$ and $\langle t \rangle$ (*cf.* Section IV.1) (Tompa *et al.*, 1987a).

Concerning the analytical implication of the kinetic approach, the mechanism of

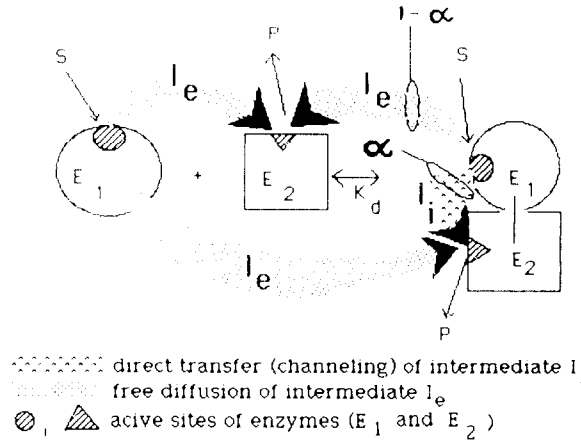



FIG. 1. Diffusion-limited and direct transfer of intermediate in the coupled reaction catalysed by $E_1$ and $E_2$.

intermediate transfer (channelling) can be identified by the relationships among macroscopic kinetic parameters (Ovádi, 1986; Orosz and Ovádi, 1987; Ovádi *et al.*, 1989). The relation of the transient time ($\tau_{app}$) for the coupled reaction catalysed by the complexed and uncomplexed enzyme species and the pseudo-first-order rate constants measured in the absence ($k_{E2}$) and presence ($k_{E2}^{E1}$) of $E_1$ are indicative of the mechanism in the interacting enzyme system (*cf.* Table 1). If the interaction of the two enzymes induces alterations in the ternary and/or quaternary structure of the enzymes without producing channelling of the intermediate the relationship described in Table 1, line 2 is fulfilled. If the intermediate produced endogenously by the $E_1E_2$ complex is channelled between the two enzymes the transient time is reduced with respect to that measured in a non-interacting system. This relationship may result from either steric hindrance which impedes the diffusion of intermediate into the bulk solution (Table 1, line 4) or simply from the juxtaposition of active sites of the enzymes in complexed form (Table 1, line 3). The latter case, which can be considered as a special one, we denoted as a leaky channel. If the escape of the intermediate from the complex is prevented the mechanism is denoted as a perfect channel (Table 1, line 5) where $\alpha = 1$ and $\langle t' \rangle \ll \langle t \rangle$. In a dynamically interacting enzyme system these kinetic parameters are composite functions of those for the processes catalysed by the complex and by the isolated enzymes.

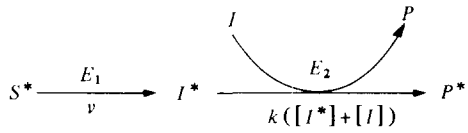
TABLE 1. RELATIONSHIPS OF THE MICROSCOPIC AND MACROSCOPIC KINETIC PARAMETERS IN ENZYME SYSTEMS

| Type of interaction | Macroscopic parameters referring to the actions of enzymes $E_1$ | $E_2$ | Microscopic parameters | Examples* | (Ref.) |
|---|---|---|---|---|---|
| 1. Non-interacting | $r_{E1} = r$ | $1/\tau_{app} = k_{E2}^{E1} = k_{E2}$ | $\alpha = 0$ | GAPD/TPI | 1 |
| 2. Interaction inducing conformational changes | $r_{E1} > r$ | $1/\tau_{app} = k_{E2}^{E1} > k_{E2}$ | $0 < \alpha < 1$ $\langle t' \rangle < \langle t \rangle$ | PFK/FBPase | 2 |
| 3. Leaky channel | $r_{E1} = r$ | $1/\tau_{app} > k_{E2} = k_{E2}^{E1}$ | $0 < \alpha < 1$ $\langle t' \rangle < \langle t \rangle$ | Ald./GAPD | 3 |
| 4. Partial channel | $r_{E1} = r$ | $1/\tau_{app} > k_{E2} > k_{E2}^{E1}$ | $0 < \alpha < 1$ $\langle t' \rangle < \langle t \rangle$ | Ald./GDH | 4 |
| 5. Perfect channel | $r_{E1} = r$ | $1/\tau_{app} \to \infty$, $k_{E2}^{E1} \to 0$ | $0 < \alpha < 1$ $\langle t' \rangle \approx 0$ | AAT/GluDH | 5 |

* GAPD = glyceraldehyde-3-phosphate dehydrogenase; TPI = triosephosphate isomerase; PFK = phosphofructokinase; FBPase = fructose-1,6-bisphosphatase; Ald. = aldolase; GDH = glycerol-3-phosphate dehydrogenase; AAT = aspartate aminotransferase; GluDH = glutamate dehydrogenase. 1: Orosz *et al.*, 1986; 2: Ovádi *et al.*, 1986; 3: Ovádi and Keleti, 1978; 4: Vértessy and Ovádi, 1987; 5: Salerno *et al.*, 1982a.

Further evidence for microcompartmentation can be obtained by adding isotope intermediate to the reaction mixture during the coupled reaction. The absence of labelling in the end-product is strong evidence for complete channelling. If the end-product is labelled in a percentage which is proportional to the degree of isotope label of the intermediate and that produced in the coupled reaction, no channelling can be assumed (*cf.* Friedrich, 1984). One can add isotope substrate to the first enzyme, "dilute" the system with non-labelled intermediate and measure the specific radioactivity of the end-product (Bryce *et al.*, 1976). The specific radioactivity of the end-product will equal unity, if complete channelling occurs and will be the function of the rate of the first and second step and of the concentration of added intermediate in the absence of or in the presence of partial channelling. The combination of isotope and kinetic methods allows us to choose between conformational change, leaky or perfect channel (Ovádi, 1986; Orosz and Ovádi, 1987) and to prove the leaky channel between aldolase and glyceraldehyde-3-phosphate dehydrogenase (Orosz and Ovádi, 1987).

The use of kinetic approach based on the comparison of the kinetic parameters, combined with isotope dilution technique enormously enhances the sensitivity of the identification of the mechanism of intermediate transfer.




If the initial substrate of the consecutive reactions is radioactive ($S^*$) and unlabelled intermediate is added to the reaction mixture then the relative specific radioactivity ($r$) of the end product ($P^*$) at a given time ($t$) can be calculated from the ratio of $[P^*]$ and the total concentration of the final product ($[P^*]+[P]$). If the measured relative specific radioactivity ($r_{meas}$) is compared with the calculated one ($r_{calc}$), assuming different models, the mechanism of intermediate transfer can be identified. For the calculations of $r$ values the equations are given in Table 2.

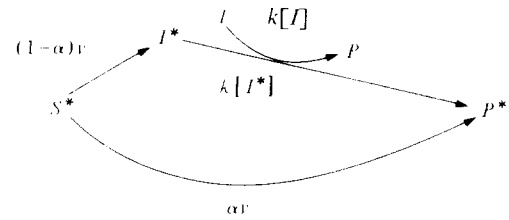
TABLE 2. SPECIFIC RADIOACTIVITY OF THE PRODUCT IN COUPLED REACTION*

| | |
|---|---|
| For non-interacting system: | $r=\dfrac{vt-v/k(1-e^{-kt})}{vt-v/k(1-e^{-kt})+[I_0](1-e^{-kt})}$ |
| For conformational changes induced by interaction: | $r=\dfrac{vt-v\tau(1-e^{-t/\tau})}{vt-v\tau(1-e^{-t/\tau})+[I_0](1-e^{-t/\tau})}$ |
| For channelling of intermediate: | $r=\dfrac{vt-v\tau(1-e^{-t/\tau})}{vt-v\tau(1-e^{-t/\tau})+[I_0](1-e^{-k't})}$ |

*$k=k_{E2}$, $\tau=\tau_{app}$ (*cf.* Table 1) and $k=k[E_2]_{free}/[E_2]_{total}$.

Obviously, this approach does not say anything about the extent of the channel, since the kinetic parameters are the weighted sums of the corresponding kinetic parameters for interacting and non-interacting enzyme species. This is because the reaction mixture always contains both complexed and free $E_1$ and $E_2$. Nevertheless, the mechanism of the interaction of functionally related enzymes can be determined without knowing the actual concentration of the heterologous complex.

If the dissociation constant is determined in an independent manner and the channelling mechanism is identified as well, then the extent of the channel can be deduced according to the following scheme and equations:




$$r_{\text{channel}} = \frac{vt-(1-\alpha)v/k'(1-e^{-k't})}{vt-(1-\alpha)v/k'(1-e^{-k't})+[I_0](1-e^{-k't})} \tag{21}$$

where $\alpha = [E_1]_{\text{bound}}/[E_1]_{\text{total}}$, (*cf.* Table 1).

Therefore, in a partially complexed enzyme system the value of the measured relative specific radioactivity is characteristic for the perfectness of the "channel", since

$$r_{\text{non-interacting}} \leq r_{\text{meas}} \leq r_{\text{channel}}.$$

(d) *Trapping methods*

One can use different "trapping" methods to prove the channelling of the intermediates of functionally related enzymes:

(i) Solvent method, by using a compound which dissolves the intermediate but not the substrate and end-product. The solvent must not inhibit either enzyme of the coupled reaction. If no or only partial channelling occurs the concentration of intermediate decreases by adding the solvent and consequently decreases the activity of the second enzyme, since first order kinetics must be assured for it (Yanofsky and Rachmeler, 1958).

(ii) Side reaction method, trapping the intermediate by a reaction which renders it unable to react with the second enzyme (Ovádi and Keleti, 1978). Its inverse is to detect the lack of such side reaction in the case of channelling (Keleti, 1978).

(iii) Enzyme probe method, to decompose specifically the intermediate by an enzyme into product which cannot react with the second enzyme if no or only partial channelling occurs (Friedrich *et al.*, 1977; Solti and Friedrich, 1979).

## III. INTERACTION OF CYTOSOLIC ENZYMES

Enzymes of glycolysis, glyconeogenesis and the pentose phosphate pathway are commonly recognized as "soluble enzymes" and many of the individual components of these and other cytosolic pathways have been intensively studied. In cytoplasm the major set of soluble enzymes belongs to glycolysis. In the yeast cell, for example, glycolytic enzymes constitute about 65% of total soluble protein (Hess *et al.*, 1979). This situation favours the protein–protein interactions in the cells (Srere, 1967). Since these dynamic macromolecular interactions are frequently loose and transient, their studies are difficult. The use of the approaches discussed in the previous chapter is widely accepted, on the one hand, to detect complex formation at physiological or even lower enzyme concentrations, on the other hand, to gain information on the functional consequences of interactions of metabolically related enzymes.

### 1. *Interactions of Purified Enzymes* In Vitro

A simple and sensitive approach used to monitor the binding of a protein to another one is measuring the signal of a covalently attached fluorescent probe. One of the earliest examples for the evaluation of quantitative data for heterologous complex formation is the aldolase–glyceraldehyde-3-phosphate dehydrogenase enzyme system. Glyceraldehyde-3-phosphate dehydrogenase was labelled with fluorescein isothiocyanate (FITC) and the anisotropy was measured as a function of aldolase concentration (Ovádi *et al.*, 1978). The

experimental points could be fitted to a model assuming a complex with an apparent dissociation constant of 0.3 $\mu$M and an apparent stoichiometry of 1:2, which probably means that tetrameric aldolase binds dimeric dehydrogenase. However, Masters and Winzor (1981) suggested that the association between aldolase and glyceraldehyde-3-phosphate dehydrogenase was artifactual, mediated by the probe introduced into the enzymes. Indeed, a fluorescein-mediated interaction of bovine serum albumin with a fluorescent derivative of prolactin has been demonstrated (Church *et al.*, 1981), i.e. the fluorescent emission spectra of the fluorescein moiety in the labelled prolactin was red-shifted and increased in intensity in the presence of albumin, However, such a phenomenon was not observed in the aldolase–dehydrogenase system; therefore, the interaction of the two enzymes *via* the fluorescent dye could be excluded. Moreover, complex formation between aldolase and glyceraldehyde-3-phosphate dehydrogenase was demonstrated by several other methods (*cf.* Table 3).

The binding of aldolase to glyceraldehyde-3-phosphate dehydrogenase was demonstrated also with yeast enzymes and the dissociation constants of the yeast enzymes, mammalian enzymes and their hybrids was compared (Batke and Tompa, 1986; Tompa *et al.*, 1986). Batke and his coworkers found that the hybrid complexes had comparable dissociation constants with that of non-hybrid ones probably due to the possible conservation of the heterologous complex-forming surfaces of these enzymes during evolution (Batke and Tompa, 1986; Tompa *et al.*, 1986).

Complex formation between aldolase and another "branching enzyme", glycerol-3-phosphate dehydrogenase had been detected many years ago. These two enzymes form cocrystals (myogen A) during isolation (Baranowski and Niederland, 1949). The first indications of the interaction between these two enzymes were provided by rapid kinetic technique and active band centrifugation (Batke *et al.*, 1980). Results of kinetic (Batke, 1978; Batke *et al.*, 1980) and fluorescence (Ovádi *et al.*, 1983) analyses showed simultaneous dependence of the specific enzymatic activity of glycerol-3-phosphate dehydrogenase and the fluorescence anisotropy of the enzyme on enzyme concentration. A model for the theoretical description of complex formation of aldolase with dissociable glycerol-3-phosphate dehydrogenase was developed (Ovádi *et al.*, 1985); moreover, the rate constants of the individual steps of complex formations were determined.

Hess and Boiteux (1972) were the first who made a systematic study of various sets of glycolytic enzymes isolated from yeast by measuring the transient time over a wide range of enzyme concentration, and they failed to detect any sign of interaction with the exception of the alcohol dehydrogenase and pyruvate dehydrogenase coupled system.

In recent years a number of authors have suggested that in certain metabolic sequences catalysed by soluble enzymes, the product of the reaction of one enzyme can be transferred directly to the subsequent enzyme via transient enzyme–enzyme interactions (*cf.* Srivastava and Bernhard, 1986a; Keleti and Ovádi, 1988). However, there are also some reports in which other authors (Grazi and Trombetta, 1980; Kvassman *et al.*, 1988; Chock and Gutfreund, 1988) re-examined the kinetics of the transfer of intermediates and suggested the original interpretations to be incorrect. For illustration of the contradictions first we discuss the aldolase–glycerolphosphate dehydrogenase system. We have demonstrated the complex formation between these two enzymes (Batke *et al.*, 1980; Ovádi *et al.*, 1983) and we analysed the mechanism of the intermediate transfer as well (Vértessy and Ovádi, 1987). However, recently *pro* and *contra* data have been published from various laboratories (Srivastava and Bernhard, 1986a; Chock and Gutfreund, 1988).

The two enzymes catalyse the conversion of fructose-1,6-bisphosphate to glycerol-3-phosphate via dihydroxyacetonephosphate formation. For the analysis of the mechanism of interaction a simple kinetic diagnostic test has been applied (*cf.* Section II.2.c). The relationship of kinetic parameters determined under interacting and non-interacting conditions indicated that the binding of exogenous intermediate (dihydroxyacetone-phosphate) to the glycerolphosphate dehydrogenase is impeded in the presence of aldolase probably by steric hindrance, whereas the endogenous intermediate produced by aldolase has direct access to the dehydrogenase within the complex. In fact, we have found that the

pseudo-first-order rate constant of the conversion of exogenous dihydroxyacetonephosphate measured in the presence of non-functioning aldolase is reduced as compared to that measured in the absence of aldolase due to an apparent increase in $K_m$ value of exogenous intermediate for dehydrogenase (Vértessy and Ovádi, 1987). Very recently, in accordance

TABLE 3. EXAMPLES OF THE *IN VITRO* OBSERVED COMPLEXES OF CYTOPLASMIC ENZYMES

| Sources of enzymes and type of the complex | Method of detection and $K_d$ (in $\mu$M) of the complexes and remarks | References |
|---|---|---|
| Rabbit muscle aldolase/GAPD* | FITC-label fluorescence anisotropy; $K_d$: 0.3 | Ovádi *et al.*, 1978 |
| | Rapid kinetics; activity of aldolase does not change in the presence of GAPD; $K_{m,GAP}$ in the presence of aldolase is decreased | Ovádi and Keleti, 1978 |
| | Complex is modulated by fructose phosphates | Neuzil *et al.*, 1989 |
| | Isotope exchange, high performance liquid chromatography; leaky channel is detected | Orosz and Ovádi, 1986, 1987; Orosz *et al.*, 1986 |
| | Kinetic analysis | Grazi and Trombetta, 1980 |
| | A "suicide" reaction | Patthy and Vas, 1978 |
| | NAD-Sepharose 4B gel-chromatography in batch system; $K_d \sim 1$ | Kálmán and Boross, 1982 |
| | Frontal gel-chromatography; complex was not found | Földi *et al.*, 1973 |
| Yeast aldolase/GAPD | FITC-label fluorescence anisotropy; $K_d \sim 0.3$ | Batke and Tompa, 1986 |
| | Hybrid complexes of yeast and muscle enzymes with the same $K_d$ is also observed | Tompa *et al.*, 1986 |
| Rabbit muscle aldolase/GDH | Cocrystallization (myogen A) | Baranowski and Niederland, 1949 |
| | Rapid reaction technique and active band ultracentrifugation | Batke, 1978; Batke *et al.*, 1980 |
| | FITC-label fluorescence anisotropy | Ovádi *et al.*, 1983 |
| | $K_d$ (ald-monomeric GDH): 1<br>$K_d$ (ald-dimeric GDH): 0.2 | Ovádi *et al.*, 1985 |
| | Channelling of DHAP in the complex | Vértessy and Ovádi, 1987 |
| | Complex is mediated by fructose phosphates | Vértessy and Ovádi, 1989 |
| Rabbit muscle aldolase/TPI | FITC-label fluorescence anisotropy and gel equilibrium; $K_d$: 2 | Salerno and Ovádi, 1982 |
| | TPI inhibits the activity of aldolase | Orosz *et al.*, 1986 |
| | GAPD and TPI compete for aldolase | Orosz and Ovádi, 1986 |
| Insect flight muscle aldolase/TPI | | Gavilanes *et al.*, 1981 |
| Rabbit liver aldolase/FBP-ase | Indication in liver | Pontremoli *et al.*, 1980 |
| | With purified enzymes FBP-ase gives a blue shift of aldolase fluorescence; Complex is specific for liver enzymes and not for muscle's | Horecker *et al.*, 1981 |
| | Limited proteolysis | Pontremoli *et al.*, 1982 |
| | Complex was not found by equilibrium and velocity sedimentation, light scattering and gel filtration, however by gel-penetration a complex with 1:1 stoichiometry was observed | Horecker *et al.*, 1981 |
| Yeast or mammalian muscle aldolase/PFK | FITC-label fluorescence anisotropy | Tompa *et al.*, 1986 |
| | Calmodulin is a modulator of the complex | Orosz *et al.*, 1987, 1988a, b |
| Rabbit brain aldolase/S100 protein | Aldolase activity is increased in the complex | Zimmer and Van Eldik, 1986 |
| Rabbit muscle PFK/FBP-ase | Mutual kinetic influence | Ovádi *et al.*, 1986 |
| | The complex is modulated by hormones and produces self-oscillation | Goldstein and Ivanova, 1987 |

TABLE 3—continued

| | | |
|---|---|---|
| Bovine brain, rabbit muscle, yeast enolase/ phosphoglycerate mutase | FITC-label fluorescence anisotropy; kinetic analysis<br>$K_d$ (for brain): 20–40<br>$K_d$ (for muscle): 0.5–2<br>$K_d$ (for hybrids of muscle/brain or yeast/brain): 0.5–2 | Batke *et al.*, 1988<br>Nazaryan and Batke, 1989 |
| GAPD/ADH and GDH/LDH | Kinetic analysis: direct transfer of NAD/HADH is assumed in the complex<br>FITC-label fluorescence anisotropy: $K_d$ (GDH/LDH) ~ 1 | *cf.* Srivastava and Bernhard, 1986a; Friedrich and Hajdu, 1987<br>Batke, 1989 |
| IsocitrateDH/GluDH | Transfer of NAD/NADH was not found | Ehrlich, 1987 |
| Yeast ADH/pyruvateDH | Transient time analysis | Hess and Boiteux, 1972 |

* Abbreviations: ADH: alcohol dehydrogenase; DH: dehydrogenase; DHAP: dihydroxyacetonephosphate; FBPase: fructose-1,6-bisphosphatase; FITC: fluoroisothiocyanate; GAP: glyceraldehyde-3-phosphate; GAPD: glyceraldehyde-3-phosphate dehydrogenase; GDH: glycerol-3-phosphate dehydrogenase; GluDH: glutamate dehydrogenase; LDH: lactate dehydrogenase; PFK: phosphofructokinase; TPI: triosephosphate isomerase.

with our results, Chock and Gutfreund (1988) reported that the aldolase is an inhibitor for the dehydrogenase-catalysed reaction since the apparent $K_m$ of dihydroxyacetonephosphate for the dehydrogenase is increased in the presence of aldolase. However, they interpreted these results in a different way. The increase of $K_m$ was attributed to the binding of substrate to aldolase which reduced the free concentration of the triosephosphate. Unfortunately, these authors did not indicate what concentrations of aldolase and substrate were applied in their experiments. However, in our experiments the concentration of aldolase was 15 $\mu$M or lower, and the concentration of dihydroxyacetonephosphate was several orders of magnitude higher; therefore, the segregation of the substrate by non-functioning aldolase can be excluded. Moreover, to increase the $K_m$ of the substrate (which is in the mM range for the dehydrogenase) by binding to aldolase, at least mM aldolase concentration would be needed, which is hardly realizable in practice. The idea of an active-site-directed interaction between aldolase and glycerolphosphate dehydrogenase is consonant with the suggestion of Srivastava and Bernhard (1986a) who observed direct transfer of the intermediate substrate from aldolase to the dehydrogenase using different kinetic approaches.

Another system which has been the subject of detailed investigations in several laboratories is the aldolase/glyceraldehyde-3-phosphate dehydrogenase system (Ovádi and Keleti, 1978; Ovádi *et al.*, 1978; Patthy and Vas, 1978; Grazi and Trombetta, 1980; Orosz and Ovádi, 1987; Kvassman *et al.*, 1988). We have found that the exogenous intermediate, glyceraldehyde-3-phosphate has the same probability to be bound to the free dehydrogenase as to the complexed one (*cf.* Table 1, line 3). However, the endogenous aldehyde form of glyceraldehyde-3-phosphate liberated at the active site of aldolase is transferred directly, at least partially, within the heterologous enzyme complex due to the proximity effect. Thus the hydration in the bulk medium of the aldehyde form generated by aldolase before reaching the active site of glyceraldehyde-3-phosphate dehydrogenase is prevented. Data of Kvassman *et al.* (1988) reported recently were consistent with ours published 10 years earlier. However, the results of their theoretical analysis have been interpreted to be compatible with a free-diffusion mechanism for transfer of glyceraldehyde-3-phosphate. In fact, the rate of the enzymatic conversion of glyceraldehyde-3-phosphate in their experiments was much higher than the hydration rate of its aldehyde form since they applied a high excess of the dehydrogenase. Under our experimental conditions the rate of the enzymatic reaction catalysed by the dehydrogenase was slower than that of the aldehyde–diol interconversion. Therefore, the unfavourable aldehyde–diol conversion of glyceraldehyde-3-phosphate, at least partly, would have occurred if the substrate were to mix with the bulk medium. This latter situation is the theoretical *sine qua non* condition for the detection of channelling complex formation in this system and this condition was not fulfilled in the experiments of Kvassman *et al.* (1988).

Channelling of the intermediate of the coupled reaction catalysed by aldolase and glyceraldehyde-3-phosphate dehydrogenase in the presence of triosephosphate isomerase was also investigated using isotope dilution technique (Orosz and Ovádi, 1987). In the presence of triosephosphate isomerase the hydration of the aldehyde form of glyceraldehyde-3-phosphate becomes negligible, since the triosephosphate exists predominantly in dihydroxyacetonephosphate form. Moreover, the excess of triosephosphate isomerase ensures a constant concentration of aldehyde form in the steady-state of the coupled reactions. In the experiment $^{14}$C-fructose-1,6-bisphosphate was used as initial substrate and unlabelled exogenous glyceraldehyde-3-phosphate was added to the system. We could show that the isotope glyceraldehyde-3-phosphate generated endogenously is more accessible for the dehydrogenase than the exogenous substrate. Therefore, the channelling of the aldehyde form of glyceraldehyde-3-phosphate has clearly been demonstrated. Moreover, the precise mechanism of intermediate transfer could be identified as "leaky channel", which refers to a situation when the exogenous intermediate binds to the complexed dehydrogenase with the same probability as to the free one. This finding indicates that the active site of dehydrogenase in its complexed form may not be blocked by aldolase. Grazi and Trombetta (1980) reported no evidence for the direct transfer of glyceraldehyde-3-phosphate between aldolase and glyceraldehyde-3-phosphate dehydrogenase on the basis of the following observation: the addition of triosephosphate isomerase decreased the rate of formation of the 3-phosphoglyceroyl enzyme intermediate similarly in both reaction systems where the two enzymes were freshly mixed or allowed to form complex. Disregarding the possibility that the heterologous complex formation during the catalysis might be very rapid, the finding of Grazi and Trombetta (1980) fits the "leaky channel" mechanism.

The reports are contradictory also for interaction between phosphoglycerokinase and glyceraldehyde-3-phosphate dehydrogenase both in respect to finding any complex formation and concerning the details of interaction (Friedrich, 1985). While gel-chromatographic and fluorimetric measurements as well as the transient time analysis with muscle enzymes by Vas and Batke (1981) proved no complex formation between these enzymes, experiments with immobilized enzymes (Ashmarina *et al.*, 1984) or kinetic approaches with halibut enzymes (Weber and Bernhard, 1982) or enzymes from pea chloroplasts (Macioszek and Anderson, 1987) led to an apparently contradictory result. Our intention is not to discuss in detail the *pro* and *contra* data (we refer to the discussion of possible sources of discrepancies by Friedrich, 1985), but rather a special attention is paid to the kinetic analysis measured at different molar ratio of phosphoglycerokinase and glyceraldehyde-3-phosphate dehydrogenase in different laboratories.

It is assumed that 1,3-diphosphoglycerate dissociates apparently very slowly (Huskins *et al.*, 1982) from 1,3-diphosphoglyceroyl-phosphoglycerokinase if the reaction is not coupled with glyceraldehydephosphate dehydrogenase (Weber and Bernhard, 1982; Sukhodolets *et al.*, 1987). Weber and Bernhard (1982) suggested that the binding of dehydrogenase to the acylenzyme facilitated the liberation of 1,3-diphosphoglycerate from kinase by a direct transfer mechanism. It has been pointed out (Friedrich, 1985; Sukhodolets *et al.*, 1987) that formally such a "one-encounter type of metabolite transfer" cannot be distinguished from the random mechanism involving free *I* by transient time measurement. Moreover, in the transient time measurements of Vas and Batke (1981) the concentration of glyceraldehydephosphate dehydrogenase was 20 times higher than that of phosphoglycerokinase. Therefore, a small fraction of the dehydrogenase could be complexed by the kinase. Accordingly, practically no change in the transient time can be expected even at high glyceraldehydephosphate dehydrogenase concentrations. However, it remains to be scrutinized, why the interaction between phosphoglycerokinase and glyceraldehydephosphate dehydrogenase is not reflected in the measurements of steady-state velocity of coupled reactions vs phosphoglycerokinase concentration in the experiments of Vas and Batke (1981). If the dissociation of 1,3-diphosphoglycerate is facilitated by the binding of dehydrogenase as suggested by Weber and Bernhard (1982) then it is not unlikely that at relatively high glyceraldehydephosphate dehydrogenase concentrations non-linear dependence of the steady-state velocity (i.e. of phosphoglycerokinase activity) on the phospho-

glycerokinase concentration should have been observed. Obviously, the kinetic effect of complex formation on the activity of phosphoglycerokinase depends on: (i) how much the dissociation of 1,3-diphosphoglyceroyl-phosphoglycerokinase is facilitated by the glyceraldehydephosphate dehydrogenase. The quantitative data are not unequivocal; and (ii) the relative concentration of the phosphoglycerokinase complexed with glyceraldehydephosphate dehydrogenase may depend on the oligomeric form of the dehydrogenase which in the experiments of Vas and Batke (1981) is predominantly in tetrameric form. The available data are insufficient to prove or disprove the reasons of the contradictory data.

The direct transfer also of NAD/NADH between alcohol dehydrogenase or lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase or glycerophosphate dehydrogenase was demonstrated (Srivastava and Bernhard, 1984, 1985, 1986a,b, 1987a,b; Srivastava *et al.*, 1985; Bernhard and Srivastava, 1987), as well as the competition of glycerolphosphate dehydrogenase and lactate dehydrogenase for NADH produced by glyceraldehyde-3-phosphate dehydrogenase (Friedrich and Hajdu, 1987). However, this type of direct coenzyme transfer seems to be absent in the system of NADP-dependent isocitrate dehydrogenase and glutamate dehydrogenase (Ehrlich, 1987) and even the original findings were recently questioned (Chock and Gutfreund, 1988). However, due to several recent reviews mentioned before we will not discuss in detail these data.

The modulation of dynamic channelling complexes of enzymes has been investigated only in a few cases. Kinetics of aldolase-catalysed conversion of fructosephosphates was analysed by coupling the aldolase reaction to the metabolically sequential enzymes, glyceraldehydephosphate dehydrogenase (Neuzil *et al.*, 1989) or glycerolphosphate dehydrogenase (Vértessy and Ovádi, 1989). At low enzyme concentrations polyethylene glycol was added to promote complex formation of aldolase and glyceraldehydephosphate dehydrogenase or glycerolphosphate dehydrogenase resulting in a significant increase in $K_m$ of fructose-1,6-bisphosphate and no change in $k_{cat}$. Gel-chromatography and fluorescence measurements showed positive modulation of the interaction of aldolase with either of the dehydrogenases by fructose-1,6-bisphosphate. While the presence of fructose-1,6-bisphosphate increased the affinity of aldolase for the dehydrogenases, the presence of neither fructose-1-phosphate nor dihydroxyacetonephosphate affected the dissociation constant of the heterologous enzyme complexes. We have concluded that the site for the binding of the C-6 phosphate group of the substrate on aldolase is likely to be involved directly or indirectly in the interactions. Since there are several similarities in the interaction of aldolase with glycerolphosphate dehydrogenase and glyceraldehydephosphate dehydrogenase, we suggested that their binding involves similar mechanisms and their binding sites on aldolase might overlap. Therefore, we suggested that fructosebisphosphate modulates the formation of channelling complex between aldolase and dehydrogenases. Obviously, the effect of metabolite levels and the alternative enzyme assemblies may mutually act upon each other. Therefore, any effect which influences the specificity of enzyme interactions may consequently result in an alteration of substrate level of the flux of metabolic pathways (Ovádi, 1988).

Another type of experiment led to the detection of complex formation between aldolase and fructosebisphosphatase which in liver catalyse successive reactions of gluconeogenesis. However, one should mention that in this case also we may find controversial data in the literature.

An early indication of an interaction between aldolase and fructosebisphosphatase came from the observation that rabbit liver aldolase tends to copurify with fructosebisphosphatase when the latter enzyme is isolated from liver of fed but not fasted rabbit (Pontremoli *et al.*, 1980). Other evidence for complex formation between aldolase and the phosphatase was provided also with purified enzymes by Horecker *et al.* (1981). They observed that the presence of fructosebisphosphatase which does not contain tryptophan caused a blue shift in the fluorescence emission spectrum of aldolase, indicating conformational changes in the aldolase molecule due to the binding of the phosphatase. Further, it was found that the amount of $Zn^{2+}$ bound to the high affinity site of fructosebisphosphatase was significantly reduced by aldolase. Changes in the conformation of fructosebisphosphatase could also be monitored by measuring its susceptibility to limited proteolysis by subtilisin in the presence

of a small molar excess of liver aldolase (Pontremoli *et al.*, 1982). It is noteworthy that each of the effects described was specific for the mixture of enzymes isolated from liver, but no similar phenomena were found with muscle enzymes.

Attempts to demonstrate the formation of a higher molecular mass complex containing liver fructosebisphosphatase and aldolase by equilibrium or velocity sedimentation, light scattering or gel-filtration have failed. However, equilibrium penetration experiments showed that the addition of liver aldolase to a solution containing liver fructose-bisphosphatase reduced the ability of the latter to enter into the interior phase of the gel. The molecular mass of the complex between the two enzymes was estimated to be approximately 300,000 Da which corresponded to a 1:1 molar complex (Horecker *et al.*, 1981). Any gross change in the catalytic properties of either enzyme induced by the other protein could not be detected. However, aldolase has been shown to undergo inactivation in liver of fasted rabbits without significant change in molecular mass or loss of its ability to bind antialdolase antibody (Pontremoli *et al.*, 1979a, 1980).

Pontremoli *et al.* (1982) reported, for both aldolase and fructosebisphosphatase, that inactivation by cathepsin M was accompanied by the loss of a segment from the C-terminus. Complex formation between the two enzymes (Botelho *et al.*, 1977; Pontremoli *et al.*, 1979b) is affected by this modification of aldolase but not by similar modification of the C-terminus of the phosphatase.

### 2. *In Search of a Multienzyme Aggregate*

Studies carried out over several years have yielded some evidence for the existence of a multienzyme complex in *E. coli* containing all enzymes of glycolysis (Macnab *et al.*, 1973; Mowbray and Moses, 1976; Moses, 1978; Gorringe and Moses, 1980). However, no other laboratories confirmed these results.

Data showing the compartmentalization of glycolysis in yeast cells (Rothstein *et al.*, 1959; Green *et al.*, 1965), in *Zygorrhyncus moelleri* (Moses *et al.*, 1959), in the endospermium of castor oil semen (Dennis and Green, 1975), in *Trypanosoma brucei* (Opperdoes and Borst, 1977; Visser *et al.*, 1981), in *Crithida fasciculata* and *Trypanosoma cruzi* (Taylor *et al.*, 1980; Aman *et al.*, 1985) has been reported. The unique compartmentation of the glycolytic pathway inside an intracellular organelle is the glycosome (Opperdoes *et al.*, 1984; Misset *et al.*, 1986; Opperdoes, 1987, 1988).

The enzymes within the glycosome influence the ATP synthesis (Hammond *et al.*, 1985) and have a half-life about one order of magnitude higher than in the cytosol (Hart *et al.*, 1987). The glycosomic enzymes—at variance with the cytosolic ones—have a marked excess of positive charges, distributed in two or more clusters about 40 Å apart, which may serve as topogenic signals for import into the glycosomes (Wiesenga *et al.*, 1987).

The possible existence of a multienzyme complex of glycolytic enzymes has been investigated also in the cytosol of muscle cells (Melnick and Hultin, 1973b). Kwon and Olcott (1965) reported the enhancement of aldolase activity of myogen by glyceraldehyde-3-phosphate dehydrogenase observed under conditions in which the dehydrogenase was enzymatically inactive.

A specific sedimentation technique to test association of glycolytic enzymes has failed to detect any significant amount of multienzyme aggregates (De Duve, 1970; Anderson and Green 1967). Clarke and Masters (1973a) reinvestigated this problem. They provided evidence in support of the occurrence of a multienzyme aggregate of glycolytic components under physiological conditions of pH and ionic strength by sedimenting a cytosol fraction (myogen) of rat muscle. It has been pointed out that the complex is very sensitive to factors such as pH, ionic strength and the concentrations of proteins and metabolites.

The compartmentalization of glycolytic enzymes (the upper and lower part of glycolysis in two separate compartments) is demonstrated in ascites tumour cells (Coe and Greenhouse, 1973), and experiments with permeabilized mouse L-929 cells indicate that none of the glycolytic enzymes can exist completely in solution, suggesting enzyme organization (Clegg and Jackson, 1988).

Several attempts are known to search enzyme–enzyme interactions within the glycolytic

pathway in erythrocytes. Many enzymes of erythrocytes have been purified and studied enzymologically. A number of hypotheses on the glycolytic control mechanism and ATP stabilization and some mathematical models of this metabolic system have appeared in the literature (Segel *et al.*, 1975; Atkinson, 1968; Rapoport *et al.*, 1974, 1976, 1977). The results suggested that any supramolecular organization of red cell glycolysis was unnecessary to describe the behaviour of the system. Nevertheless, a number of studies have proposed that many of the classical "cytoplasmic" proteins of the cell exist in ordered structures which may include interactions with membranous elements of the cell.

Early observations suggesting some interaction between cell membrane and soluble enzymes came from the study of incorporation of inorganic phosphate into glycolytic intermediates in erythrocytes. Since ATP was labelled faster by $^{32}P_i$ than was intracellular $P_i$, some authors suggested that glyceraldehyde-3-phosphate dehydrogenase is located at or in the membrane both in erythrocytes (Gourley, 1952; Prankerd and Altman, 1954; Gerlach *et al.*, 1958; Latzkovits *et al.*, 1972), and in HeLa cells (Niehaus and Hammerstedt, 1976). The binding of soluble enzymes by the erythrocyte membrane was further suggested by the localization of some glycolytic enzymatic activity on the membrane fraction after hypotonic haemolysis (Green *et al.*, 1965; Schrier, 1966, 1967; Mitchell *et al.*, 1965; Arese *et al.*, 1974). The experimental data are summarized in Table 4.

TABLE 4. ENZYME–MEMBRANE INTERACTIONS IN ERYTHROCYTES

| | | |
|---|---|---|
| Enzyme bound to the membrane through band-3 protein | Glyceraldehyde-3-phosphate dehydrogenase* | Kant and Steck, 1973; McDaniel and Kirtley, 1975; McDaniel *et al.*, 1974; Solti and Friedrich, 1976; Shin and Carraway, 1973; Girotti, 1976; Letko and Bohnensack, 1975; Allen *et al.*, 1987 |
| | Aldolase | Green *et al.*, 1965; Solti and Friedrich, 1976; Strapazon and Steck, 1976, 1977; Yeltman and Harris, 1980; Wilson *et al.*, 1982 |
| | Phosphofructokinase | Karadsheh and Uyeta, 1977; Higashi *et al.*, 1979 |
| | 3-Phosphoglycerate kinase | Green *et al.*, 1965; Schrier, 1966; Tillmann *et al.*, 1975; Braun and Kirtley, 1977 |
| Enzymes located near the cell membrane detected by the molecular sieving property | Glyceraldehyde-3-phosphate dehydrogenase, Lactate dehydrogenase, Phosphoglycerate kinase | Cseke and Szabolcsi, 1983; Cseke *et al.*, 1978; Szabolcsi and Cseke, 1981 |
| Saponine-induced release from intact cell | Glyceraldehyde-3-phosphate dehydrogenase | Kliman and Steck, 1980 |
| Autoradiographic localization of $^{3}$H-iodoacetate labelled enzyme | Glyceraldehyde-3-phosphate dehydrogenase | Solti *et al.*, 1981 |
| Crosslinking in intact erythrocyte | Glyceraldehyde-3-phosphate dehydrogenase | Yeltman and Harris, 1980; Keokitichai and Wrigglesworth, 1980 |
| NMR | Glyceraldehyde-3-phosphate dehydrogenase | Wilson *et al.*, 1982 |

* The glyceraldehyde-3-phosphate dehydrogenase-binding site of bovine band-3-protein is at a distance of molecular mass $\approx 5000$ Da from its N-terminal end (Moriyama and Makino, 1987). However, the physiological binding of glyceraldehyde-3-phosphate dehydrogenase to the erythrocyte membrane was questioned on the basis that it was observable only at low ionic strength (Maretzki *et al.*, 1974; Fujii and Sato, 1975; Maretzki *et al.*, 1989).

Experimental data strongly suggest that there is a supramolecular structural organization at least of the upper part of glycolysis under the erythrocyte membrane (Salhany and Gaines, 1981; Friedrich, 1984). Such localization is further suggested by the compartmentation of glycolytic intermediates in the erythrocytes, as tested by the experiments with an enzyme-

probe (Friedrich *et al.*, 1977; Solti and Friedrich, 1979). The structural network of erythrocytes including the band-3-protein and spectrin–actin reticulum is probably responsible for the formation of enzyme–enzyme complexes of the glycolytic pathway near the membrane, which on the other hand causes the compartmentation of intermediates (Friedrich, 1974; Murthy *et al.*, 1981; Haest, 1982).

### 3. *Interactions of Enzymes with Structural Elements*

#### (a) *Interaction of soluble cytosolic enzymes with membranes*

Mitochondrial membranes can also bind enzymes. Mitochondrial membrane in brain was the first shown to bind hexokinase (Crane and Sols, 1953; Knull *et al.*, 1970; Wilson, 1978), but the same phenomenon was demonstrated in liver (Rose and Warms, 1967; Felgner *et al.*, 1979), heart (Font *et al.*, 1975), small intestine (Mayer and Hübscher, 1971) and ascites tumour cells (Rose and Warms, 1967). The binding of hexokinase to the mitochondrion activates the enzyme and effects metabolite channelling (Gots *et al.*, 1972; Wilson, 1972; Gots and Bessman, 1974; Viitanen and Geiger, 1979). Brain hexokinase binds to porin (a binding protein of the mitochondrial outer membrance which concomitantly forms the contact points between the outer and inner membranes, *cf.* Jancsik *et al.*, 1988) and the hexokinase–porin complex is located in a cholesterol-free membrane domain (Dorbani *et al.*, 1987).

It is also theoretically conceivable that the kinetic and regulatory properties of membrane-adsorbed (soluble) enzymes are different from those of the free (soluble) forms (Kurganov and Loboda, 1979; Kurganov *et al.*, 1978). Experimentally the cytosolic glycerolphosphate dehydrogenase was found to bind reversibly to synthetic and mitochondrial membranes (Jancsik and Horváth, 1984) and to be regulated by the adsorption–desorption phenomenon (Jancsik and Keleti, 1986). Similarly, glyceraldehyde-3-phosphate dehydrogenase may be bound to liposomes, the result of which is the change of the conformation of the enzyme (Michalak *et al.*, 1987). Lactate dehydrogenase also associates with cellular structures and the different isoenzymes have different adsorption properties (Güttler and Clausen, 1967; De Domenech *et al.*, 1970; Ehman and Hultin, 1973).

Binding of glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerokinase to sarcolemmal and sarcoplasmic reticular membranes has been demonstrated. The binding was found to be reversible, charge-dependent and inhibitory (Pierce and Philipson, 1985).

Glycolytic and other cytoplasmic enzymes associate also with the membranes of the particulate fraction of lysed nerve endings. Hexokinase and fumarase are associated with mitochondrial membrane. Aldolase, glucosephosphate isomerase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase show enrichment in fractions containing synaptosomal membranes (Knull, 1978). Glucose-phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, aldolase, pyruvate kinase and lactate dehydrogenase became fixed when intact synaptosomes were incubated with glutaraldehyde. The immobilized enzymes are located near the synaptosomal membrane, perhaps in association with actin found at this site (Knull, 1980). Indeed, subcellular distribution of aldolase, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase in brain parallels that of brain actin (Tamir *et al.*, 1972; Clarke and Masters, 1973b; Blitz and Fine, 1974; Clarke and Morton, 1982), and they are bound, as is phosphofructokinase, to the particulate fractions (Clarke and Masters, 1972).

About 40% of hexokinase, 10% of glyceraldehyde-3-phosphate dehydrogenase, 8% of pyruvate kinase and 1% of 3-phosphoglycerate kinase is associated with the cytoplasmic side of the plasma membrane of glioma cells (Daum *et al.*, 1988).

#### (b) *Interactions with skeletal elements and cell particles*

The first findings showing the interaction of cytosolic enzymes with muscle proteins was published in the late 1960s and early 1970s (Karpatkin, 1967; Arnold and Pette, 1968, 1970; Melnick and Hultin, 1973a; Clarke and Masters, 1976). The apparent molecular mass of

some glycolytic enzymes is increased due to their binding to the proteins of contractile apparatus (Clarke and Masters, 1973a, 1974a; Földi *et al.*, 1973). The adsorption seems to be reversible and depends upon pH and ionic strength (Arnold *et al.*, 1971), as well as on the nature of the soluble enzyme (Arnold *et al.*, 1969), the structure of the individual isoenzymes (Masters and Holmes, 1975; Masters *et al.*, 1987) or on the concentration of specific metabolites (Clarke and Masters, 1975). Correlation exists between the phylogenetic stability of actin and that of several glycolytic enzymes in muscle and other tissues (Pollard and Weihing, 1974; Anderson *et al.*, 1969; Perham, 1969; Cohen *et al.*, 1973). On the other hand, in the presence of $Mg^{2+}$, the formation of actin filaments is hindered by glyceraldehyde-3-phosphate dehydrogenase and this effect is counteracted by KCl required for their correct formation (Lanzara and Grazi, 1987). Phosphofructokinase and filamentous actin form a specific association as demonstrated by electron microscopy and this reversible association may play a role in regulating the enzymatic activity and consequently the glycolysis during metabolic acidosis (Roberts and Somero, 1987). Not only actin but also troponin and tropomyosin must be considered as potential binding sites for glycolytic enzymes (Walsh *et al.*, 1977), and the binding depends on ionic strength (Clarke and Masters, 1975; Walsh *et al.*, 1980).

Moreover, it was found that the phosphorylation may play a role in the regulation of phosphofructokinase since the phosphorylated form of the enzyme has a higher apparent affinity for F-actin than does the non-phosphorylated form (Luther and Lee, 1986). F-actin acts as a positive effector of the phosphorylated form and the effect of F-actin is specific. These results seem to be consistent with *in vitro* observation which shows that upon stimulation of muscle contraction, the enzyme is phosphorylated to a greater extent and the binding to muscle matrix is increased.

Interactions of soluble enzymes with skeletal elements can lead to the formation of compartments for glycolytic enzymes and intermediates (Ottaway, 1979; Kurganov *et al.*, 1985; Ureta, 1985), which was found in rat diaphragm (Shaw and Stadie, 1957, 1959; Landau and Sims, 1967; Kalant and Breitner, 1971), rat skeletal muscle (Dully *et al.*, 1969), rat liver (Threlfall and Heath, 1968) and mouse ascites cells (Moses and Lonberg-Holm, 1966).

The thin filament F-actin–tropomyosin–troponin binds strongly glyceraldehyde-3-phosphate dehydrogenase, aldolase, phosphofructokinase, lactate dehydrogenase, pyruvate kinase, glucose-6-phosphate dehydrogenase but weakly triosephosphate isomerase, phosphoglycerate kinase, phosphoglycerate mutase, enolase and hexokinase (Clarke and Masters, 1975). It is interesting to note that troponin-T and glyceraldehyde-3-phosphate dehydrogenase share a common antigenic determinant (Sanders *et al.*, 1987). Association between thin filaments of muscle and aldolase was demonstrated by electron microscopy (Morton *et al.*, 1977), ultracentrifugal analysis (Clarke *et al.*, 1974) and affinity chromatography (Bronstein and Knull, 1981). The experimental results obtained with F-actin–tropomyosin are best described by a model in which there is one aldolase binding site per heptameric repeat unit on the filament (Masters *et al.*, 1981).

The binding of aldolase to actin increases the susceptibility of aldolase to proteolytic attack by trypsin or chymotrypsin (Dedman *et al.*, 1975). The binding of aldolase (or aldolase + glyceraldehyde-3-phosphate dehydrogenase) to the thin filaments of glycerinated rabbit psoas muscle produces a significant change in their small angle X-ray diffraction pattern (Stewart *et al.*, 1979). Aldolase crosslinks the filaments of F-actin or F-actin–tropomyosin and F-actin–tropomyosin–troponin as shown by electron micrographs of their paracrystals (Stewart *et al.*, 1980). The extent of aldolase and glyceraldehyde-3-phosphate dehydrogenase bound to actin filament is increased by electrical stimulation of anaerobic muscle (Clarke *et al.*, 1980). Under physiological conditions aldolase should be regarded as an equilibrium mixture of free and myofibril-bound forms, the relative proportions of which are a function of the metabolic state of the cell (Kuter *et al.*, 1983; Harris and Winzor, 1985, 1987).

Recently, Walsh and Knull (1988) reported that PEG enhanced several glycolytic enzyme–F-actin, enzyme–myogen and purified enzyme–enzyme interactions. These interactions, in which one protein may associate with any of several other proteins, including the

enzyme–enzyme–F-actin interaction and depicted by Clarke as "piggy-backing" (Clarke *et al.*, 1983) are consistent with the microtrabecular lattice structure.

One of the potentially most significant factors affecting metabolic regulation in the cellular microenvironment may be the interaction between enzymes and cellular structures (Masters, 1977, 1978; Kuter *et al.*, 1981). For example, binding of aldolase to F-actin–tropomyosin–troponin filaments produces major alterations in the kinetic parameters and renders them $Ca^{2+}$-sensitive (Arnold and Pette, 1970; Walsh *et al.*, 1977). Similarly, the kinetic parameters of glyceraldehyde-3-phosphate dehydrogenase, phosphofructokinase and lactate dehydrogenase are altered by adsorption to muscle proteins (Dagher and Hultin, 1975; Pette, 1978; Liou and Anderson, 1980; Eronina *et al.*, 1975; Sugrobova *et al.*, 1983).

Recently, the existence of a glycolytic minicomplex in the muscle and the facilitated binding of triosephosphate isomerase to myofibrils when both aldolase and glyceraldehyde-3-phosphate dehydrogenase were bound to the filament was demonstrated (Stephan *et al.*, 1986). Glyceraldehyde-3-phosphate dehydrogenase and phosphofructokinase have distinct active sites for actin, as revealed by proteolysis (Humphreys *et al.*, 1986). Interaction of actin and xanthine oxidase was also demonstrated (Lanzara *et al.*, 1988).

Aldolase can be bound also to the microsomes in the muscle and this interaction results in an increase of the Michaelis constant for fructose-1,6-bisphosphate (Weiss *et al.*, 1981). Similarly, lactate and malate dehydrogenase is bound to the microsomal fraction in chicken liver (Sagristá and Bozal, 1987) and rat brain (Franco *et al.*, 1988). The glycolytic enzymes are bound to particulate fraction also in carrot and sugar beet (Moorhead and Plaxton, 1988). Glyceraldehyde-3-phosphate dehydrogenase associates with mono- and polyribosomes and is one of the three major RNA-binding proteins of reticulocytes (Ryazanov, 1985; Ryazanov *et al.*, 1987, 1988).

The enzyme binds to microtubules and this binding modulates the enzymatic activity and quaternary structure (Durrien *et al.*, 1987a,b). Muscle aldolase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and muscle-type lactate dehydrogenase have been found to be bound as well to the cytoskeletal tubulin as to actin (Karkhoff-Schweizer and Knull, 1987) suggesting that the model of the microtrabecular network involving the association of soluble enzymes with actin filaments may need to be slightly modified.

Very recently a model has been developed which proposes that the glycolytic sequence is best described as consisting of a number of segments *in vivo*, each segment formed by a cluster of isozymes (*cf.* Ureta, 1978), many of which can interact with the actin filaments. Such segmentation and compartmentation are suggested to play a role in meeting the different types of energy requirement in the cytoplasm of divergent cell types (Masters *et al.*, 1987). This model based on the adsorption of enzymes on subcellular structures has been extended by the "direct transfer" hypothesis of Bernhard (Ryazanov, 1988). The basic idea is that the binding of metabolites results in desorption of the enzymes from subcellular structures during each catalytic act which makes the enzymes mobile and capable of directly transferring metabolites to the other enzymes.

## IV. INTERACTIONS OF MITOCHONDRIAL ENZYMES

### 1. *Citric Acid Cycle and Some Closely Related Enzymes*

Before the 1970s it was generally believed that the enzymes of the citric acid cycle were dispersed randomly in the mitochondrial matrix (for reviews, *cf.* Srere, 1972; Srere and Mosbach, 1974), but later it was suggested that proteins in it interact as multienzyme complexes (Srere and Henslee, 1980; Srere, 1980; Glund and Walther, 1982; Förster, 1988). This idea has been based on the following observations:

(i) The experimentally observed rate of the Krebs cycle is much higher than the calculated rate of the citrate synthase reaction. In other words, the apparent mitochondrial concentration of free oxaloacetate is low ($4 \times 10^{-8}$ M) and since the $K_m$ for oxaloacetate of rat liver citrate synthase is about $4 \times 10^{-6}$ M, citrate synthase would express only about 1% of its maximal activity, which is too low to account for the observed rate of respiration of liver mitochondria (Halper and Srere, 1977). To

reconcile this apparent discrepancy the existence of a complex of citrate synthase and malate dehydrogenase was proposed by Srere (1972). In such a complex the channelling of oxaloacetate may result in the observed high rate of the Krebs cycle.

(ii) Oxaloacetate is a key metabolite also of other metabolic pathways in mitochondria, it regulates the activity of succinate dehydrogenase and the aspartate–malate shuttle; further, it is a starting point for gluconeogenesis. Thus channelling of oxaloacetate is likely (Marco *et al.*, 1974) since the organization of the citric acid cycle enzymes would create a special microenvironment and in this way maintain a high flux through the cycle (Halper and Srere, 1977; Beeckmans and Kanarek, 1981).

(iii) Srere (1980) concluded that the concentration of enzymes in rat liver mitochondrial matrix is over 50% by weight and a direct consequence of this is that these enzymes probably exist and behave as a multienzyme complex rather than as enzymes in solution. Recent experiments with gently disrupted rat liver mitochondria suggest that the tricarboxylic acid cycle exists as a sequential complex of enzymes, a metabolon, *in situ* (Srere, 1985; Robinson *et al.*, 1987; Lyubarev and Kurganov, 1987). Moreove, linear relationships were found between mitochondrial forces and cytoplasmic flows. These findings are suggestive of extensive enzyme organization within these metabolic pathways (Berry *et al.*, 1987).

Several publications appeared in the last 10 years indicating the existence of physical interactions between the enzymes of the citric acid cycle and other metabolically related enzymes (*cf.* Table 5 and references therein). Five enzymes (i.e. aspartate aminotransferase, malate dehydrogenase, citrate synthase, pyruvate dehydrogenase complex and glutamate dehydrogenase) may have exceptional importance in this respect.

(i) Interaction of aspartate aminotransferase and malate dehydrogenase: Backman and Johansson (1976) assumed that complexes between aspartate aminotransferase and malate dehydrogenase on both sides of the membrane might be involved in the regulation of $NAD^+$/NADH ratio (*cf.* also the review of Dawson (1979)). The original finding on the channelling of oxaloacetate between aspartate aminotransferase and malate dehydrogenase (Bryce *et al.*, 1976) was questioned (Manley *et al.*, 1980). More recently a large citric acid cycle–(malate dehydrogenase–aspartate aminotransferase shuttle) multienzyme complex has been indicated by Beeckmans and Kanarek (1981). This complex was assumed to have physiological implications on the regulation of metabolic fluxes by channelling oxaloacetate towards the citric acid cycle or towards the aspartate–malate shuttle.

(ii) Interaction of malate dehydrogenase and citrate synthase: Malate dehydrogenase catalyses the only reaction of the citric acid cycle with a rather unfavourable equilibrium, [L-malate]/[oxalate] = $10^4$ (Newsholme and Start, 1973). This equilibrium forces the cell to remove oxaloacetate rapidly in order to ensure the running of the cycle. On the other hand, citrate synthase, the enzyme next to malate dehydrogenase, is considered to be the main control point of the cycle (Newsholme and Start, 1973; Srere, 1974). Therefore Beeckmans and Kanarek (1981) reckoned "that besides extensive control by different substances on this enzyme itself, the fact of switching on and off a physiological interaction between malate dehydrogenase and citrate synthase would be an extra way of regulating the cycle activity especially with the equilibrium of the former reaction lying far to the left."

The apparent dissociation constant of the complex of mitochondrial citrate synthase and mitochondrial malate dehydrogenase is $10^{-6}$ M while that of the complex of mitochondrial citrate synthase and the cytoplasmic malate dehydrogenase is $1.5 \times 10^{-5}$ M (Tompa *et al.*, 1987b).

(iii) Interaction of pyruvate dehydrogenase complex with other enzymes: Pyruvate dehydrogenase complex is also considered as a regulator at the switch point between energy metabolism and gluconeogenesis (Hucho, 1975). As a result of the close functional relationship between pyruvate dehydrogenase complex and citrate synthase it was assumed there is an interaction between them (Sümegi *et al.*, 1980; Sümegi and Alkonyi, 1983). They found that the $K_m$ value of CoA on the pyruvate

TABLE 5. INTERACTIONS OF MITOCHONDRIAL ENZYMES (CITRIC ACID CYCLE AND CLOSELY RELATED ENZYMES)

| Enzyme complex | Remarks | References |
|---|---|---|
| Binary-enzyme complexes: | | |
| Malate dehydrogenase–citrate synthase (MDH–CS)* | | Srere, 1974; Fahien *et al.*, 1979; Bryce *et al.*, 1976 |
| | Activation of malate dehydrogenase by citrate synthase | Tompa *et al.*, 1987b |
| | Channelling of oxaloacetate | Datta *et al.*, 1985 |
| | Polyethylene glycol induced formation of the enzyme complex | Merz *et al.*, 1987 |
| MDH–fumarase | Fumarase–MDH is considered as an anchor to succinate dehydrogenase (located in mitochondrial inner membrane, Ernster and Kuylenstierna, 1970) giving connection between citric acid cycle and respiratory chain | Beeckmans and Kanarek, 1981 |
| MDH–aspartate amino transferase (AAT) | The complex is detected by counter current distribution only between the mitochondrial enzymes | Backman and Johansson, 1976; Dawson, 1979; Fahien *et al.*, 1979; Beeckmans and Kanarek, 1981; Fahien and Kmiotek, 1983 |
| AAT–glutamate dehydrogenase (GLuDH)† | Interaction of pig liver mitochondrial enzymes was demonstrated by gel-chromatography and steady state fluorescence anisotropy | Fahien *et al.*, 1979; Fahien and Kmiotek, 1983; Salerno *et al.*, 1975 |
| | Bovine liver GLuDH and pig heart cytoplasmic AAT interact especially in the presence of NADH plus ammonium ion or NAD plus aspartate | Fahien and Smith, 1969, 1974 |
| | The complex is stabilized by bifunctional crosslinker dimethyl-3,3′-dithiobis-propion-imidate | Hucho *et al.*, 1975; Fahien *et al.*, 1978 |
| | Detection of complex by analysing the consecutive reaction. Data are fitted to a model of direct transfer of the intermediate assuming 1:1 stoichiometry in binding of GLuDH and AAT | Salerno *et al.*, 1982a |
| | Complex of beef liver enzymes was indicated by time-resolved fluorescence anisotropy | Churchich and Lee, 1976 |
| | Coprecipitation | Salerno *et al.*, 1982a |
| Citrate synthase–thiolase | | Sümegi *et al.*, 1985 |
| Citrate synthase–pyruvate dehydrogenase complex (PDC) | The $K_m$ value of CoA on PDC and that of acyl-CoA on CS is decreased in the complex | Sümegi *et al.*, 1980; Sümegi and Alkonyi, 1983 |
| α-Ketoglutarate dehydrogenase (KGDH)–succinate thiokinase | | Porpáczy *et al.*, 1983 |
| KGDH–NAD-dependent isocitrate dehydrogenase | | Porpáczy *et al.*, 1987 |
| KGDH–NADH: ubiquinone oxidoreductase (UQORase) | | Porpáczy *et al.*, 1987 |
| UQORase–PDC | | Sümegi and Srere, 1984 |
| UQORase–MDH | | Sümegi and Srere, 1984 |
| UQORase–β hydroxyacyl CoA dehydrogenase | | Sümegi and Srere, 1984 |
| β hydroxyacyl CoA dehydrogenase specific binding protein in inner mitochondrial membrane | | Kispál *et al.*, 1986 |

TABLE 5—continued

| | | |
|---|---|---|
| GLuDH–MDH | | Fahien *et al.*, 1979 |
| CS–AAT | | Fahien *et al.*, 1979; Fahien and Kmiotek, 1983 |
| Ternary-enzyme complexes: | | |
| MDH<br>–acetyl CoA carboxylase<br>–phosphoenolpyruvate carboxylase | In *Euglena gracilis* this complex participates in $CO_2$ fixation | Wolpert and Ernst-Fonberg, 1975a,b |
| Fumarase–MDH AAT | | Beeckmans and Kanarek, 1981 |
| Fumarase MDH CS | | Beeckmans and Kanarek, 1981 |
| MDH–CS–lactate dehydrogenase | Immobilized three-enzyme system. A model for micro-environmental compartmentation in mitochondria | Srere *et al.*, 1973 |
| MDH–AAT KGDH | MDH activity is enhanced due to decrease in $K_m$ of malate<br>α-Ketoglutarate and citrate enhance the dissociation of MDH from this complex | Fahien *et al.*, 1988 |

* $K_d$ (in absence of substrates) = 1 μM; $K_d$ (in presence of α-KG) = 0.2 μM; $K_d$ (in presence of NADH) = 5 μM; $K_d$ (of complex of mitochondrial CS and cytoplasmic MDH) = 15 μM; Tompa *et al.*, 1987b.

† $K_d$ = 8.6 μM; Salerno *et al.*, 1982a.

$K_d$: apparent dissociation constant assuming 1:1 stoichiometry in the complex. KG: α-ketoglutarate.

dehydrogenase and that of acyl-CoA on citrate synthase decreased in the coupled system when compared to those of the individual reactions.

(iv) Interaction of aspartate aminotransferase and glutamate dehydrogenase: Complexes between glutamate dehydrogenase and mitochondrial aminotransferases may even play a physiologically significant role in converting amino acids to keto acids plus ammonium ions in liver mitochondria. This statement is based on the following reasons: (a) glutamate dehydrogenase, alanine aminotransferase and aspartate aminotransferase are in the same mitochondrial compartment (De Rosa and Swick, 1975; Sottocasa *et al.*, 1963); (b) in liver mitochondria the level of glutamate dehydrogenase is even higher (Schmidt *et al.*, 1963; Klingenberg *et al.*, 1965) than that required *in vitro* to form a complex with mitochondrial ornithine, alanine or aspartate aminotransferase (Fahien and Smith, 1974; Fahien *et al.*, 1977); (c) complexes between glutamate dehydrogenase and these mitochondrial aminotransferases are quite active *in vitro* in catalysing an amino acid dehydrogenase reaction even in the absence of α-ketoglutarate or glutamate (Fahien and Smith, 1974; Fahien *et al.*, 1977); (d) the reductive amination of oxaloacetate could occur more efficiently through the consecutive reaction catalysed by the complex of aspartate aminotransferase and glutamate dehydrogenase than by the free enzyme and within the complex practically complete channelling was observed (Salerno *et al.*, 1982). The efficiency ($\alpha$) of the intermediate transfer (*cf.* also Section II.2.c) in this complex was determined recently (Tompa *et al.*, 1987a) and experimental values could be fitted by using $\alpha = 0.9$ and $\langle t' \rangle = 0$.

Considering the rather great and continuously increasing number of observed enzyme–enzyme interactions presented above in (i)–(iv) (*cf.* also Table 5), to answer the question about the hierarchy (specificity) of the possible complex formation (in competing cases) as well as about the effect of various intermediates and other factors becomes especially important in respect of *in vivo* functions of these complexes. Unfortunately, although many individual observations and data are known, we did not possess a general outline till now. However, some evidence concerning the specificity of complex formation and the regulation of complex association/dissociation by intermediates suggests the possible role of enzyme enzyme interactions even in *in vivo* conditions.

Mitochondrial malate dehydrogenase and aspartate aminotransferase can form hetero-

complexes with either glutamate dehydrogenase (Fahien *et al.*, 1979) or citrate synthase. As indicated by Fahien and Kmiotek (1983) the complexes with aspartate aminotransferase are favoured over those with citrate synthase. At low enzyme concentration the only detectable complex is that of aspartate aminotransferase and glutamate dehydrogenase.

NADPH disrupts the complexes with malate dehydrogenase but has little effect on the complexes with aspartate aminotransferase. Oxaloacetate disrupts the complexes with citrate synthase but has hardly any effect on the complexes with glutamate dehydrogenase. NADH + malate abolish the complexes of malate dehydrogenase and glutamate dehydrogenase, aspartate aminotransferase and glutamate dehydrogenase, malate dehydrogenase and citrate synthase but favour the formation of the complex of aspartate aminotransferase and citrate synthase.

$NAD^+$, oxaloacetate, citrate, ATP, L(−)- or D(+)-malate had no effect on the association of citrate synthase and malate dehydrogenase, whereas α-ketoglutarate increased and NADH decreased it. On one hand, α-ketoglutarate, as a Krebs cycle intermediate, also has alternate metabolic fates, it is involved in glutamate formation or in the malate–aspartate shuttle. The activation of malate dehydrogenase by citrate synthase indicated by Tompa *et al.* (1987b) is consistent with the necessity of maintaining balanced fluxes between α-ketoglutarate dehydrogenase and citrate synthase (*cf.* Williamson and Cooper, 1980). On the other hand, since NADH affects the strength of the association between citrate synthase and malate dehydrogenase, the degree of enzyme interaction (and hence the oxaloacetate–citrate flux) may be readily controlled by changes in the $NAD^+$/NADH ratio. Accordingly, the $NAD^+$/NADH ratio may serve a dual role. First, it is a major determinant of the energy-generation flux in the Krebs cycle by virtue of the regulatory effects on various enzymes therein (Williamson and Cooper, 1980). In addition, it may function in the control of metabolic flow of carbon moiety between catabolism and anabolism.

Bovine serum albumin, alpha-globulin, ovalbumin and lactate dehydrogenase did not alter the nanosecond and/or steady-state fluorescence polarization of labelled mitochondrial aspartate aminotransferase. Similar results for the specificity of aspartate aminotransferase–glutamate dehydrogenase complex were obtained by precipitation experiments in polyethylene glycol solution (Fahien and Kmiotek, 1979).

Other proteins (bovine serum albumin, citrate synthase, cytoplasmic malate dehydrogenase) did not coprecipitate with either glutamate dehydrogenase or aspartate aminotransferase. The coprecipitation of aspartate aminotransferase and glutamate dehydrogenase was markedly decreased by increasing either the pH and/or ionic strength (Salerno *et al.*, 1982b) or by addition of ligands such as malate plus NADPH, NADH or GTP (Fahien and Kmiotek, 1983).

Other observations also indicate that formation of enzyme–enzyme complexes can be influenced by substrates (Fahien *et al.*, 1977; *cf.* Table 5). It was found that in the presence of NADPH and ammonium ions glutamate dehydrogenase can react with the pyridoxal-phosphate form of aspartate aminotransferase to produce the pyridoxaminephosphate form of this enzyme and NADP (Fahien and Smith, 1974; Fahien *et al.*, 1971; Shemise *et al.*, 1972). The affinity of aspartate aminotransferase to glutamate dehydrogenase can be enhanced in the presence of NADP plus glutamate or aspartate (Fahien and Smith, 1974).

Interaction between bovine liver glutamate dehydrogenase and pig heart cytoplasmic aspartate aminotransferase could also be observed especially in the presence of NADH plus ammonium ions or NAD plus aspartate (Fahien and Smith, 1974).

### 2. *Other Mitochondrial Enzymes*

Mitochondrial creatine phosphokinase is compartmentalized (Bessman *et al.*, 1978; Erickson-Viitanen *et al.*, 1982a,b) and coupled not only to oxidative phosphorylation (Erickson-Viitanen *et al.*, 1982a) but also to nucleotide translocase (Brooks and Suelter, 1987). The carbamoyl phosphate metabolism and the arginine pathways in the mitochondria of *Neurospora crassa* are compartmentalized by channelling (Wasternack, 1984; Davis, 1986; Davis and Ristow, 1987). Moreover, in *Saccharomyces cerevisiae* carbamylphosphate synthetase associates with aspartate transcarbamylase (Belkaïd *et al.*, 1987; Penverne and

Hervé, 1983) and as a consequence of this complex formation carbamylphosphate is channelled between the two enzymes (Belkaïd *et al.*, 1988).

## V. CONTROL OF METABOLISM BY MACROMOLECULAR INTERACTIONS

For the description of regulatory properties of metabolic pathways a control analysis has been developed (Higgins, 1963, 1965; Savageau, 1972, 1976; Savageau *et al.*, 1987a,b; Kacser and Burns, 1973, 1979; Kacser, 1983; Heinrich and Rapoport, 1973, 1974a,b, 1983; Heinrich *et al.*, 1977; Newsholme and Crabtree, 1973, 1976; Crabtree and Newsholme, 1985). Kacser (1983) has suggested the idea of "molecular democracy" to characterize each enzyme in a metabolic process as an autonomous entity and the control as a sort of linear superposition of the effects of the individual enzymes. The milieu of this "molecular society" is a bulk aqueous solution with non-interacting enzymes and non-compartmentalized metabolites homogeneously dispersed therein. The links in such a metabolic network are the intermediate metabolite pools.

### 1. *Control of Metabolism in Bulk Medium*

Assuming a chain of unsaturated but reversible enzymes carrying out the overall conversion of external substrate $X_1$ to the final external product $X_2$ via successive intermediary metabolites $S_1, S_2, \ldots S_{n-1}$

$$X_1 \xrightarrow{E_1} S_1 \xrightarrow{E_2} S_2 \longrightarrow \cdots S_{n-1} \xrightarrow{E_n} X_2$$

it follows (Kacser and Burns, 1973) by using the term of kinetic power (Keleti and Vértessy, 1986; Welch *et al.*, 1988) that the overall flux, $F$, for this system is:

$$F = ([X_1] - [X_2]/K_{eq,1}K_{eq,2} \ldots K_{eq,n})/(1/k_{\Gamma,1} + 1/k_{\Gamma,2}K_{eq,1} + 1/k_{\Gamma,3}K_{eq,1}K_{eq,2} + \cdots). \quad (22)$$

The factor describing the response of the overall rate to an infinitesimal change in enzyme concentration is the control coefficient (Kacser and Burns, 1973; Heinrich and Rapoport, 1974a). The manner by which the control of a homogeneous, bulk-phase metabolic process is shared amongst all of the enzymes is revealed from the Summation Theorem (Kacser and Burns, 1973, 1979) which shows that the sum of all control coefficients of a given metabolic pathway equals unity.

In the metabolic control the control coefficients of the individual enzymes are connected by the Connectivity Property (Kacser and Burns, 1973, 1979; Heinrich and Rapoport, 1974a; Heinrich *et al.*, 1977). The Elasticity Coefficient (Kacser and Burns, 1973, 1979; Heinrich and Rapoport, 1974a; Heinrich *et al.*, 1977) reveals the relative infinitesimal change of enzyme velocity in the function of relative infinitesimal change in substrate concentration.

The Control Coefficient, Elasticity Coefficient, Connectivity Property and Summation Theorem are the most important parameters of metabolic control.

Metabolic cycles and cascades have an important role in the amplification mechanism of enzyme activity and thus in the control of metabolic pathways (Newsholme and Crabtree, 1973, 1976; Crabtree, 1976; Newsholme *et al.*, 1984; Goldbeter and Koshland, 1982; Sorribas and Bartrons, 1986) similarly to the futile cycles (Hue, 1982). In this type of bulk medium metabolic control, the kinetic properties of the enzymes in the pathway are related to the extent to which the same enzymes control the steady-state concentration of metabolites (Westerhoff and Chen, 1984). The calculation of the flux control coefficients can be performed from the kinetic parameters by using a simple matrix algebra procedure (Fell and Sauro, 1985) which was extended to allow determination of the concentration control coefficients (Sauro *et al.*, 1987) even if moiety conserved cycles (Hofmeyr *et al.*, 1986) are involved in the pathway. Hofmeyr (1986) elaborated the method of steady-state modelling of metabolic control. The theory was extended to branched metabolic systems (Heinrich *et al.*, 1987; Giersch, 1988c; Small and Fell, 1989) and to parallel systems working with isoenzymes or alternative enzymes (Derr and Derr, 1987).

The control theory was successfully applied to the glycolysis of erythrocytes (Rapoport *et al.*, 1976; Heinrich *et al.*, 1987) or of some streptococci (Poolman *et al.*, 1987), to the

conversion of glucose in rat liver extract (Torres *et al.*, 1986), the gluconeogenetic pathway (Groen *et al.*, 1986), the aromatic amino acid metabolism in isolated rat liver cells (Salter *et al.*, 1986), the arginine pathway of *Neurospora crassa* (Flint *et al.*, 1981), mitochondrial respiration, oxidative phosphorylation and translocator action (Groen *et al.*, 1982a,b, 1983; Tager *et al.*, 1983; Wanders *et al.*, 1984a,b; Mazat *et al.*, 1986), the electron flux through cytochrome oxidase (Murphy and Brand, 1987), serine biosynthesis in mammals (Fell and Snell, 1988) and interrelation between glycolysis and hexose monophosphate shunt in erythrocytes (Schuster *et al.*, 1988). The possibility of the application of control theory to central metabolic pathways of *E. coli* (Holms, 1986, 1987), to microbial growth and metabolism in general (Kell and Westerhoff, 1986; Kell, 1987), and to the Calvin photosynthesis cycle (Pettersson and Ryde-Pettersson, 1988) was discussed.

### 2. *Control of Metabolism in Organized Systems*

The previously discussed bulk-phase, "pool" view of metabolic control may be valid for some enzymatic process *in vivo*; however, it is not valid for many others. In the previous sections it was shown that there is now abundant evidence that much—perhaps the majority—of cellular metabolism is spatially organized on a scale much smaller than that of any of the well-known organelles. This organization includes membrane-bound enzyme clusters, multienzyme complexes and enzyme arrays attached to the cytomatrix. While our present knowledge of the actual microenvironments in these organized enzyme regimes is very limited, it is quite apparent that the thermodynamic and kinetic character of the reaction–diffusion flow therein is distinctly different from the condition in an aqueous bulk-phase solution, where the only links in a metabolic network are homogeneous pools of substrates and products. Consequently, the cell metabolism is more likely controlled in a manner of "supramolecular socialism" (Welch and Keleti, 1987) where the "cytosociological" behaviour of the enzymes is manifested in the evolutionarily governed formation of multienzyme systems (Welch and Keleti, 1981).

The kinetic consequence of supramolecular organization of the enzymes is manifested most effectively if channelling occurs (Hess and Wurster, 1970; Welch, 1977b; Bartha and Keleti, 1979; Easterby, 1981, 1986; Keleti, 1984, 1986b; Wasternack and Hause, 1986; Tompa *et al.*, 1987a; Hofer *et al.*, 1987; Ovádi *et al.*, 1989; Keleti *et al.*, 1988). The existence of intracellular microenvironments demands us to modify our concepts about cell metabolism and its control traditionally based on simplistic bulk-phase view.

The relationships for the velocity of the individual enzymes in the pathway and for the flux in organized systems remain formally identical with those derived in the case of the bulk-phase system; however, the meaning of the constants will be different (Welch *et al.*, 1988).

The flux-control coefficients for an organized multienzyme system represent the change in the kinetic properties of each enzyme due to its interaction with the next one in the pathway (Welch *et al.*, 1988).

The flux itself depends on the state of organization of the enzyme system. If the organized system entails localized "pools" of metabolites and enzymes, the structural constraints in such microenvironments might impart a vectorial character onto the metabolic flow therein, along with an anisotropic distortion of the local concentration–diffusion fields. For tightly interacting multienzyme complexes which channel metabolites, the "pool" concept for intermediate substrates does not apply (Welch *et al.*, 1988).

The kinetic parameters of metabolic control for organized multienzyme systems were formulated and the control analysis for organized heterogeneous enzyme systems was presented recently (Keleti and Vértessy, 1986; Welch *et al.*, 1988; Keleti, 1989b). The difference between the bulk-phase and organized systems was proved for the control coefficient (Crabtree and Newsholme, 1987, 1988; Giersch, 1988a) and the connectivity relation (Giersch, 1988b). It is reasonable to stress that enzymes behave differently providing they are in free solution or in a living cell (*cf.* Kacser and Porteous, 1987), since due to the compartmentalization of both enzymes and metabolites, enzyme catalysis and consequently metabolic pathways are vectorial processes (Kell and Westerhoff, 1985). Moreover, the fixed charges of biological membranes attract or repulse mobile ions (among them $H^+$) and

consequently the concentration of these ions which modulate enzymatic activity will be different in the vicinity of the membranes and in the bulk-phase. Therefore, metabolic pathways organized by one or more enzymes associated with membranes are controlled differently than those in the bulk medium (Ricard and Noat, 1984; Ricard, 1989).

If the catalytic capacity of an enzyme in the cell is much higher than that of the other enzymes which react with the same substrates, then it will bring its own reaction partners very close to thermodynamic equilibrium. This is called "near-equilibrium relation" (Holzer *et al.*, 1956; Bücher and Klingenberg, 1958; Hohorst *et al.*, 1959; Krebs and Veech, 1969; Veech *et al.*, 1969, 1970). Since the kinetic requirements for a near-equilibrium relation are not too restrictive it can be expected that whole pathways may consist of them (Reich, 1976). If we assume the whole metabolic pathway in near-equilibrium, the $K_{eq}$s defined as the equilibrium constants between the intermediate pools will be simply the thermodynamic equilibrium constants of the reversible reactions catalysed by the respective enzymes.

The equation of the flux (eqn (22)) in this case will have the form:

$$F=\left(X_1 \prod_{i=1}^{n} k_{\Gamma,i}^{f} - X_2 \prod_{j=1}^{n} k_{\Gamma,j}^{r}\right) \Big/ D_c \tag{23}$$

where

$$D_c = \prod_{i=2}^{n} k_{\Gamma,i}^{f} + k_{\Gamma,1}^{r} \prod_{i=3}^{n} k_{\Gamma,i}^{f} + \prod_{j=1}^{2} k_{\Gamma,j}^{r} \prod_{i=4}^{n} k_{\Gamma,i}^{f} + \prod_{j=1}^{3} k_{\Gamma,j}^{r} \prod_{i=5}^{n} k_{\Gamma,i}^{f} + \cdots + \prod_{j=1}^{n-1} k_{\Gamma,j}^{r} \tag{24}$$

and superscripts $f$ and $r$ refer to the forward and reverse reaction, respectively.

The rates in a near-equilibrium system depend only on kinetic power and metabolite concentrations. The flux depends on the concentration of the first substrate and last product in the whole pathway and on the kinetic power of all enzymes. The control coefficients depend only on kinetic powers.

According to the rules of reversible Michaelis-Menten mechanisms (Keleti, 1986c) the relation of the individual kinetic constants of the enzymes with the kinetic parameters of metabolic control will be different depending on whether the reversible reaction reaches the steady-state at infinite time (diffusion controlled reactions) in the forward and reverse reactions or the kinetics in one direction is rapid equilibrium (Keleti, 1989b).

For complex metabolic networks as organized, bifurcating and oscillating systems, etc., complex mathematical formulations are elaborated (Savageau, 1969a,b, 1970, 1971a,b, 1985; Liao and Lightfoot, 1987; Palsson *et al.*, 1985; Palsson and Lightfoot, 1985a,b; Palsson and Groshans, 1988; Bohnensack, 1985).

### 3. *Thermodynamics of Metabolic Control*

The thermodynamic control theory was elaborated by Westerhoff *et al.* (1987a). The free energy elasticity coefficient is defined as

$$\varepsilon_S^i = \{\partial \ln|v_i|/\partial \ln[S]\}_{[P],[E]} = \{\partial \ln|v_i|/\partial \ln(\mu_S/RT)\}_{\mu,[E]} = \varepsilon_\mu^i \tag{25}$$

where $\mu_S$ is the chemical potential of the substrate.

Relationships exist between flux control coefficients and free energy elasticity coefficients. This approach can also resolve some problems in the bulk medium control analysis caused by compartmentation. The thermodynamic control theory was successfully applied to the control of mitochondrial respiration by the adenine nucleotide translocator (Westerhoff *et al.*, 1987b).

In order to obtain a better physical feel for the channelling process, we explore more deeply the apparent first-order rate constant, the reciprocal of transient time within an enzyme complex.

In the formalism of the absolute rate theory in a channelled system the reciprocal transient time is a real first-order rate constant, which is directly related to the standard activation free-energy of the respective coupled reaction (Keleti, 1986b; Keleti *et al.*, 1988) and is

characteristic of the rate-limiting intramolecular conversion $I \rightarrow P$ within the bienzyme complex. As such, it (like all first-order rate constants for the transformation of enzyme-bound intermediates) is constrained by the theoretical upper limit specified by the universal frequency $k_B T/h$ ($=6.21 \times 10^{12}$ sec$^{-1}$ at 298 K) (Keleti *et al.*, 1988).

Since the bulk-phase form of the reciprocal transient time is only an apparent first-order rate constant, the transition-state theory cannot be applied, in general, to the coupled reaction involving the separated enzymes in bulk solution (i.e. where there is no intramolecular product formation). However, if the rate of this coupled reaction is diffusion-controlled, then $1/k_D[E_2]_T$ (where $k_D$ is the Smoluchowski diffusion-rate coefficient) sets the lower limit for the bulk-phase transient time (Keleti and Welch, 1984).

In conclusion, we can quantitate the kinetic (transition-state) barrier for the coupled enzymatic conversion $I \rightarrow P$ in the two extreme cases as follows (Keleti *et al.*, 1988). We have

$$\Delta G_c^{\#} = -2.48 \ln[(h/k_B T)/\tau] \tag{26}$$

in kJ/mol at 298 K, for the first-order case of perfect channelling within the bienzyme complex, $E_1E_2$, where $\tau$ is the transient time within the complex. For the diffusion-limited, second-order reaction of $I$ and $E_2$, occurring in a system of non-interacting enzymes, we find

$$\Delta G_D^{\#} = -2.48 \ln(k_D/\Phi'). \tag{27}$$

The transition-state free energy, $\Delta G_D^{\#}$, represents the barrier for diffusive particle motion in the liquid state, and $\Phi'$ is a pro-pre-exponential factor (Keleti, 1983), whose form depends on the particular model of the liquid state (Glasstone *et al.*, 1941; North, 1964; Hill, 1985).

Obviously, the association/dissociation of multienzyme complexes may have direct consequences on the overall energy profile of coupled reactions (Keleti, 1986b; Srivastava and Bernhard, 1986b; Keleti *et al.*, 1988; Bernhard, 1988).

However, one should be aware that the validity of thermodynamic arguments of Albery and Knowles (1976) for balancing changes in basic free energy in a metabolic pathway catalysed by reversible enzymes depends on kinetic factors and is not general (Kamp *et al.*, 1988).

## VI. CONCLUDING REMARKS

Studies on purified enzymes have contributed a good deal to the understanding of the catalytic action of several enzymes at the molecular level. However, the results obtained with enzymes taken out from their natural milieu do not necessarily demonstrate how the enzymes function *in vivo*. The structural and functional organization of enzymes can be influenced by the presence of other enzymes and macromolecules as well. This validates the search for enzyme–enzyme interactions. Many examples presented previously demonstrate that the catalytic efficiency of the enzymes involved in consecutive reactions is increased if the enzymes are clustered and organized into multienzyme complexes. Enzyme–enzyme interactions may occur either in tight complexes or in weak ones among so-called soluble enzymes in different compartments of the cell, like cytosol, mitochondrial matrix, etc. Compartments for soluble enzymes are furnished by intracellular structures or elements of the contractile system. In addition, microcompartments for metabolites can be formed in the channels of complexes of functionally related enzymes. The simultaneous existence of interactions between enzymes, structural elements and metabolites may ensure a dynamism of the weak complexes. This dynamism manifests itself in the association–dissociation of the complexes resulting in the alternation of catalytic efficiency or regulatory behaviour.

The existence of dynamic microcompartments may ensure alternative vectorial motions of metabolites, a means for the coordination of metabolic pathways of the same intermediates. In this way the living cell spares energy by preventing undesirable side reactions of intermediates. Vice versa, the metabolites themselves influence the state of the dynamic microcompartments. In other words, the metabolic control of heterogeneous organized enzyme systems differs from that of enzymes in homogeneous bulk medium.

The choice of interactions, complexes, compartments we have dealt with in this review

reflects mainly our special interest and certainly does not give a comprehensive picture covering all ramifications of this rapidly developing field.

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## NOTE ADDED IN PROOF

The theories concerning metabolic control have been reviewed recently (Meléndez-Hevia *et al.*, 1987; Torres *et al.*, 1988a; Cornish-Bowden, 1989a,b; Sorribas and Savageau, 1989a,b,c). The control analysis was extended for convergent metabolic pathways (Torres *et al.*, 1988b), and for homologously (subunit–subunit) and heterologously (enzyme–enzyme, enzyme–cytomatrix) interacting organized systems (Kacser, 1989, Kacser *et al.*, 1989; Welsh and Keleti, 1989) including the role of external ligands (Khodolenko, 1988). The matrix method for the calculation of the parameters was further developed (Reder, 1988; Cascante *et al.*, 1989a,b) and the graph method elaborated (Hofmeyr, 1989). The transit time and metabolic flux control analyses were combined (Acerenza *et al.*, 1989; Easterby, 1989; Torres *et al.*, 1989). Further data are presented on the supramolecular organization of metabolic pathways (Kurganov, 1988; Lyubarev and Kurganov, 1989) including metabolic compartmentation (Spivey and Merz, 1989; Batke, 1989a,b; Ovádi, 1989) and multienzyme complexes in the one-carbon metabolism (Schieh and Strong, 1989).

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