

Snapshots of three intermediates at the active site of pyruvate oxidase

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Identifying the structures of transient intermediates is an essential step in the elucidation of an enzymatic reaction mechanism. Cryocrystallography reveals the structures of three thiamine diphosphate derivatives as intermediates in the action of pyruvate oxidase.

In all living cells, thiamine is essential in energy metabolism. Thiamine diphosphate (ThDP) is an essential cofactor for the metabolism of pyruvate, the end product of the glycolytic pathway; for the metabolism of α -ketoglutarate, a central intermediate in the tricarboxylic acid pathway; and for the conversion of fructose-6-phosphate and xylulose-5-phosphate, intermediates in carbohydrate metabolism. ThDP also unlocks the chemistry of other processes in plant and microbial metabolism, including the production of acetolactate for amino acid biosynthesis and the decarboxylation of oxalyl coenzyme A in oxalate metabolism. Decades ago, bioorganic chemists unmasked essential aspects of the chemistry of ThDP and postulated rational chemical mechanisms for its biological functions¹. In this issue, Tittmann and co-workers uncover the structures of three ThDP intermediates at the active site of pyruvate oxidase and show the ways these are correlated with the theory of ThDP action².

Chemical synthesis of the compounds 2-(α -hydroxyethyl)-ThDP, 2-(α,β -dihydroxyethyl)-ThDP and 2-acetyl-ThDP (AcThDP) opened the door to testing the prevailing theories about the action of ThDP. Two lines of evidence proved that the compounds were chemically competent as intermediates. First, the authentic compounds were shown to be identical to intermediates produced by ThDP-dependent enzymes³⁻⁷. Further,

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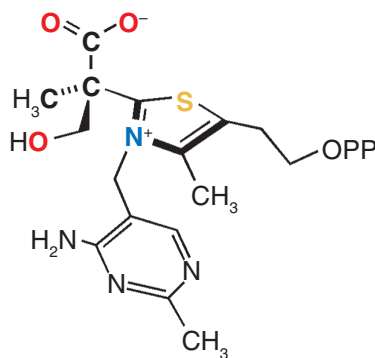


Figure 1 S-LThDP. The structure of POX with LThDP bound at the active site reveals the configuration at the optical center at C2 of the lactyl group in the S configuration, as it exists in the enzymatic site. P, phosphate. Boldface atoms define the stereocenter.

these compounds were transformed by the enzymes into the expected products. The next advance came when Tittmann and co-workers performed transient kinetic analyses to observe the kinetic behavior of the presumed intermediates. Their rapid mix-quench NMR experiments demonstrated the transient production of several of the synthetic compounds as reaction intermediates. These included 2-(α -hydroxyethyl)-ThDP, AcThDP and a species that seemed to be 2-lactyl-ThDP (LThDP), which had not previously been synthesized⁸.

The ThDP- and flavin-dependent pyruvate oxidase (POX) catalyzes the conversion of pyruvate, O_2 and inorganic phosphate (P_i) into acetyl phosphate, CO_2 and H_2O_2

by a mechanism that is not fully understood but involves several ThDP intermediates. The current study by Wille and associates reports the X-ray crystal structures of POX with three of the postulated ThDP intermediates bound at the active site¹. Wille and colleagues used clever biochemical strategies and cryocrystallography to obtain these structures. S-LThDP is the dominant form at the steady state owing to rate limitation by decarboxylation, so that crystals that are soaked with substrate and frozen contain this intermediate. Reduction of the flavin by an external reducing agent blocks electron transfer within POX, so that 2-(α -hydroxyethylidene)-ThDP accumulates and is observed in crystals frozen after addition of pyruvate. Exclusion of phosphate blocks acetyl transfer and allows AcThDP to accumulate and be observed in the frozen crystals. One of the intermediates, LThDP, has not been observed before at an enzymatic site, and details of its structure are revealed here for the first time, including its absolute stereochemical configuration. Moreover, the conformation of the carboxyl moiety turns out to be stereoelectronically ideal for the next step in the mechanism, the decarboxylation of this intermediate.

The structures of AcThDP and 2-(α -hydroxyethylidene)-ThDP at the active site are also described, as well as that of a phosphonate analog of LThDP. LThDP has an optical center at C2 of the lactyl moiety. Synthetic LThDP would be racemic, but active sites generally are asymmetric and likely to accommodate only one stereoisomer. This is borne out by the structure of the POX-LThDP complex². The structure shows S-LThDP at the active site, with the carboxylate

plane positioned perpendicular to the plane of the thiazolium ring (Fig. 1).

Other ThDP-dependent enzymes that process pyruvate also generate LThDP as the predecarboxylation species. It will be interesting to see whether they also generate the S stereoisomer of this new intermediate. LThDP is presumed to be an intermediate in the reactions of pyruvate with other ThDP-dependent enzymes, including pyruvate decarboxylase, pyruvate dehydrogenase,

acetylacetyl synthase, pyruvate:ferridoxin oxidoreductase, the flavin-independent pyruvate oxidase and other pyruvate oxidoreductases. If these enzymes are all part of the same evolutionary family, one might expect S-LThDP to be a common intermediate within the family. However, the only way to determine whether this is true is by application of the biochemical and crystallographic methods used in the study of Wille and associates².

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Signalomic signatures enlighten drug profiling

Robert T Abraham

When introduced into living cells, drugs frequently evoke unanticipated responses that are due either to off-target effects or to previously unknown interactions between the intended target and other biochemical pathways. The development of a panel of high-resolution sentinel assays for signal-transduction cascades in human cells promises to enhance the power of chemical genetics and increase the efficiency of drug-discovery research.

Small-molecule drugs are developed with the singular intent of modulating the function of a molecular target implicated in the etiology of a specific human disease. However, the overall phenotypic response elicited by most drugs reflects both the ‘on-target’ interaction with the protein of interest and also a variable number of ‘off-target’ interactions involving other, usually undefined cellular components. Though ‘off-target’ effects can sometimes confer a therapeutic advantage^{1,2}, they frequently give rise to undesirable side effects that may derail the development of the drug candidate. In most cases, researchers optimize lead candidates for their on-target activities while having only a vague understanding of the intrinsic and/or emergent off-target liabilities of the compounds under development. On page 329 of this issue, MacDonald *et al.*³ describe a high-content, cell-based assay strategy that may allow chemists to exclude undesirable chemical classes early in the discovery process and optimize their lead candidates with a far more comprehensive perspective on any off-target liabilities incurred during the lead-candidate optimization process.

The protein-fragment complementation assay (PCA) strategy used in this study takes

advantage of the extensive reliance of signal-transduction pathways on constitutive or inducible interactions among component proteins. To detect changes in protein partnering, MacDonald and co-workers individually fused proteins to a complementary fragment of an intensely fluorescent variant of yellow fluorescent protein (YFP) and expressed both members of this protein pair in human embryonic kidney 293 (HEK293) cells (Fig. 1a). Fusion-protein dimerization in the transfected cells juxtaposes the complementary YFP fragments and reconstitutes the active fluorophore. The high fluorescence yield of the enhanced YFP reporter allows detection of dimerization reactions in stably transfected cells expressing biologically relevant levels of each fusion protein. Cell imaging with an automated, high-content fluorescence microscope provides both quantitative and spatiotemporal information about the protein-protein interaction itself, and it also provides an indirect readout for the status of the signaling pathway in which the protein pair resides. Drugs that interfere with the assembly of an active YFP reporter (either directly, or indirectly by blocking an upstream step in the signaling pathway) induce a decrease in total cellular fluorescence. Drugs that exert more subtle effects, such as alterations in the subcellular localization of the reporter complexes, can also be scored by high-content image analysis.

Although the PCA concept has been implemented previously^{4–6}, MacDonald *et al.*³

have pushed this technology to a new level by constructing a diverse set of parallel PCAs in HEK293 cells (one PCA per transfected cell line). The 49 PCA assays in this panel cover a broad spectrum of cytoplasmic and intranuclear signaling pathways. Using these assays as pathway sentinels, the authors examined a panel of 107 compounds associated with several distinct therapeutic areas. The summed PCA results yield a ‘signalomic signature’ for each drug, and hierarchical analysis of the results allows clustering of compounds based on their overall activity profiles (Fig. 1b). As predicted, the PCAs faithfully grouped drugs with similar chemical structures and/or identical molecular targets, for example β -adrenergic receptor agonists. The predictive power of these sentinel PCAs was established with the demonstration that the statins, which inhibit cholesterol biosynthesis by blocking 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, clustered together in spite of the fact that their actual molecular target was absent from the PCA panel. Furthermore, the PCA assays revealed that chemically similar agonists for the nuclear hormone receptor PPAR- α manifested distinct profiles of off-target activities. This finding could lead to discoveries of previously undetected or ‘hidden’ phenotypes³ that are provoked by well-established therapeutic agents.

Cell proliferation is a highly complex phenotypic response that is orchestrated by a

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