A steady-state kinetic analysis of nucleoside diphosphatase activity of Golgi membranes

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Introduction

Nucleoside diphosphatase (NDPase; EC 3.6.1.6) catalyses the dephosphorylation of a nucleoside diphosphate to a nucleoside monophosphate and inorganic phosphate. Although several authors reported the purification of NDPase from a variety of sources [1–5], little is known about the steady-state kinetics of NDPase activity of Golgi membranes.

Several studies have demonstrated that NDPase of isolated membranes is only active in the presence of added cations [6–8]. Kuhn & White [8] showed the NDPase of rat mammary gland to be most effectively stimulated by Ca²⁺ and to a lesser extent by Mg²⁺, Mn²⁺ or Co²⁺. Brandan & Fleischer [6] showed rat liver Golgi NDPase was also maximally stimulated by Ca²⁺, but not by Mg²⁺, Mn²⁺ or Co²⁺. In contrast to the results of Kuhn & White [8] stimulation by Ca²⁺ appeared to follow Michaelis–Menten kinetics. Sano et al. [4] showed Mn²⁺ to be most effective in stimulating activity. In view of these contradictions, uncertainties and the lack of detailed kinetic analysis, we undertook a series of experiments to investigate the steady-state kinetics of NDPase activity of Golgi membranes.

Abbreviation used: NDPase, nucleoside diphosphatase.

Materials and methods

All chemicals were purchased from the Sigma Chemical Company, Poole, U.K. except polyvinyl alcohol (average molecular mass 14 kDa) which was bought from the Aldrich Chemical Company Inc., Milwaukee, U.S.A. Subcellular fractions enriched in Golgi apparatus were prepared from lactating mammary glands of guinea-pigs by the method of Morre [9] as modified by Smith & Brew [10]. The NDPase was assayed as described by James et al. [7]. For reactions catalysed by the NDPase of Golgi membranes there are effectively two substrates: the cation and the nucleoside diphosphate. Kinetic analysis was carried out by measuring initial velocities at varying concentrations of one substrate in the presence of a series of fixed concentrations of the second substrate and vice versa. Inhibitors of NDPase activity were found and subsequently used to investigate the binding order of the two substrates to the enzyme.

Velocity data as a function of substrate and inhibitor concentrations were fitted directly by the appropriate rate equations using non-linear least-squares regression methods, with FORTRAN 77 programs and the Numerical Algorithms Group Library of subroutines running on the Amdahl 5860 computer. Initial velocity data for each substrate variation was also displayed as Lineweaver-Burk plots.

Results and discussion

Earlier work in this laboratory has shown that NDPase activity was not measurable in the absence of added cation and that Ca²⁺ was found to stimulate the highest activity regardless of tissue/species used [7]. This agrees with the results of Kuhn & White [8] and Brandan & Fleischer [6].
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(a) Double-reciprocal plot showing the relationship between the initial reaction velocity \( v \) and the concentration of UDP at different fixed concentrations of Ca\(^{2+}\); (b) Double-reciprocal plot showing the relationship between the initial reaction velocity \( v \) and the concentration of Ca\(^{2+}\) at different fixed concentrations of UDP. Velocities expressed as nmol of phosphate released/min per mg. Concentrations in mM.

Mg\(^{2+}\), Mn\(^{2+}\) and Co\(^{2+}\) also stimulated activity, but to a lower degree (20–70% compared with 90–95% shown by Kuhn & White [8]). The activation of NDPase by Ca\(^{2+}\) was shown to follow Michaelis-Menten kinetics [6].

A steady-state kinetic investigation of the NDPase activity of Golgi membranes was initiated by estimating initial velocities at varying concentrations of Ca\(^{2+}\) in the presence of a fixed concentration of UDP and vice versa. The results of this experiment are shown in Fig. 1. The results are most consistent with the sequential two-substrate initial velocity equation below which describes a sequential rather than a ping-pong mechanism:

\[
V = \frac{V_m A B}{k_4 + k_1 A + k_2 B + AB}
\]

where \( V_m \) is maximum velocity, \( v \) is velocity of reaction, \( A \) is substrate 1 concentration, \( B \) is substrate 2 concentration, \( k_4 \) is the Michaelis constant for \( A \) and \( k_2 \) the Michaelis constant for \( B \).

Preliminary experiments indicated that Ba\(^{2+}\) and sulphate were relatively effective inhibitors of NDPase activity. These inhibitors were used to determine the binding order of the substrates to the enzyme. Inhibition of NDPase was investigated by carrying out a set of experiments where several concentrations of the inhibitor were used with several concentrations of Ca\(^{2+}\), the UDP concentration remaining unchanged and vice versa. Ba\(^{2+}\) inhibits competitively with respect to Ca\(^{2+}\) and non-competitively with respect to UDP. Sulphate inhibits non-competitively with respect to UDP, but competitively with respect to Ca\(^{2+}\). These results suggest that the NDPase of Golgi membranes possibly follows an ordered sequential mechanism in which the Ca\(^{2+}\) binds first to the enzyme followed by the UDP. The proposed kinetic mechanism is illustrated below. This is the first time a kinetic mechanism for NDPase activity has been proposed.

\[
E \rightleftharpoons E \cdot Ca^{2+} \rightleftharpoons E \cdot Ca^{2+} \cdot UDP \rightarrow \text{Products}
\]

Dead-end complex


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Acetazolamide-resistant carbonic anhydrase activity with expansion of the vascular bed in tonic skeletal muscle of rats

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Mammalian skeletal muscle fibres contain carbonic anhydrase (CA; EC 4.2.1.1) in the sarcoplasm, in mitochondria and in membrane-bound forms associated with the sarcolemma and sarcoplasmic reticulum. Suggested functions have included facilitation of CO\(_2\) diffusion, involvement in maintenance of intracellular acid-base homeostasis, and the provision of H\(^+\) and HCO\(_3^-\) ions for transmembrane exchanges. Inhibition of CA in isolated rat soleus muscles by chlorazolamide or by sodium cyanate results in intracellular acidosis, reduced isometric tension in response to direct stimulation, and prolonged relaxation times [1]. In rat muscle the low-activity but relatively sulphonamide-resistant iso-enzyme (CA III) is uniformly distributed in the sarcoplasm to which it is confined, principally in the type I fibres predomi-