Structure and regulation of Escherichia coli isocitrate dehydrogenase

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There has recently been great interest in the regulation of the isocitrate dehydrogenase (NADP-linked, EC 1.1.1.42) (ICDH) from Escherichia coli. Growth on acetate involves a metabolic branch-point at the level of isocitrate between the citric acid cycle and the glyoxylate bypass, which provides the precursors necessary for biosynthesis. Bennett & Holmes [1, 2] showed that ICDH was partially, reversibly inactivated during growth on acetate. The work of Garnak & Reeves [3, 4] indicated that the inactivation of ICDH resulted from its phosphorylation. Since then, the phosphorylation of ICDH has been studied intensively in several laboratories (see [5] for review). It is now accepted that the major, if not the only, role of this phosphorylation is to apportion flux between the citric acid cycle and the glyoxylate bypass during growth on acetate. In this paper, we focus mainly on the effects of phosphorylation on the conformation and activity of the ICDH from E. coli ML308.

The active, unphosphorylated form of ICDH was isolated from E. coli ML308 grown on glycerol; the protein represented about 0.6% of the total soluble protein of the cell [6]. The final purification step involved elution of the enzyme from a Procion Red-Sepharose column with NADP'. Physicochemical characterization revealed that ICDH is a dimer of identical subunits of M, 45,000 [6]. Product-inhibition studies showed that the enzyme obeys a compulsory-order steady-state mechanism, with coenzyme binding first [7].

LaPorte & Koshland [8] discovered that both the phosphorylation and the dephosphorylation of ICDH are catalysed by a single, bifunctional enzyme, now termed ICDH kinase/phosphatase. The kinase activity transfers the γ-phosphoryl group of ATP to one serine residue per subunit of ICDH, and this inactivates the enzyme almost completely [9, 10]. The phosphatase activity has one unusual property, namely that it absolutely requires either ATP or ADP for activity [9, 10]. It catalyses the release of P, from phosphorylated ICDH, and can reactivate the enzyme completely provided that the kinase is not active under the incubation conditions [10]. The regulatory properties of ICDH kinase/phosphatase and its role in controlling the phosphorylation state and activity of ICDH are relatively well understood (e.g. 5). The current model of the organization of the enzyme, based on studies of its ATPase activity [11] and photoaffinity labelling experiments [12], is that the kinase and phosphatase activities share the same or overlapping sites. It has been suggested [11] that the phosphatase reaction represents the kinase back-reaction obligatorily coupled to ATP hydrolysis.

In the majority of phosphorylation systems that have been studied heretofore, phosphorylation affects the affinity of the target enzyme for a substrate or effector rather than the Vmax. Yet phosphorylation inactivates ICDH essentially totally under Vmax assay conditions [9, 10]. How does this occur? We now present our current hypothesis for the molecular mechanism underlying this effect.

Our attempts to purify ICDH from E. coli cells grown on acetate resulted in the isolation of a form of the enzyme that was essentially devoid of catalytic activity [6]. In agreement with the results obtained from phosphorylation with ICDH kinase in vivo (see above), this inactive enzyme differed chemically from the active form only in that it contained one phosphate group per subunit. The inactive ICDH, unlike the active enzyme, was unable to bind to Procion Red-Sepharose [6]. Active ICDH binds to this column via its NADP-binding site, as judged by the fact that it is eluted from the column specifically and quantitatively with a low concentration of NADP'. We therefore suggested that inactive ICDH was inactive precisely because it was unable to bind NADP [6].

We tested this idea by studying the binding of NADPH to ICDH fluorometrically [13]. Additionally, we used NADP and NADPH to reassociate ICDH resulted in a significant enhancement of the fluorescence of the coenzyme, with no change in its emission and excitation maxima. Titration experiments showed that NADP binds very tightly to active ICDH, with a stoichiometry of approximately one per subunit. In contrast, addition of NADPH to inactive ICDH had no effect on the fluorescence yield or spectrum of the coenzyme. This study, and limited proteolysis experiments (see below), suggested very strongly that inactive ICDH is indeed unable to bind coenzyme [13].

We used limited proteolysis in an attempt to detect conformational changes in ICDH elicited by the binding of NADP* [13]. We found that both phosphorylation of and binding of NADP* to active ICDH dramatically lowered the resistance of the enzyme to proteolysis, indicative of the occurrence of conformational changes. Moreover, the changes induced by these two very different treatments were remarkably similar, in that both phosphorylation and binding of NADP markedly stabilize a 'nicked' form of the enzyme against further proteolytic attack. In contrast to the active enzyme, addition of NADP* to phosphorylated ICDH did not affect its susceptibility to proteolysis [13].

To explain these results, we suggested that phosphorylation of ICDH occurred close to, or at, its NADP-binding site [13, 14]. We assume that the NADP-binding site contains one or more positive charges that interact with the phosphate groups of the coenzyme; this charge-charge interaction could trigger the conformational change induced by binding of NADP* (see above). The introduction of a phosphate group on a serine residue close to the coenzyme-binding site could trigger a similar conformational charge, and would be expected to block binding of coenzyme, exactly as is observed (see above). This hypothesis is illustrated schematically in Fig. 1. Our recent work has been aimed at

Abbreviation used: ICDH, isocitrate dehydrogenase.

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investigating this hypothesis in more detail, and in studying the nature of the interaction between ICDH and ICDH kinase.

We first addressed the question of the nature of the positively charged group(s) at the coenzyme-binding site of ICDH [15]. The arginine-specific reagent phenylglyoxal inactivates ICDH rapidly in a pseudo-first-order process. Both NADP⁺ and isocitrate can protect the enzyme against inactivation. The effect of isocitrate is, at first sight, surprising in view of the fact that the enzyme obeys an ordered mechanism with NADP⁺ binding first [7]. The most likely explanation of this anomaly is that, since the last step in the purification involves elution with NADP⁺, the enzyme preparation actually contains small amounts of the coenzyme. Use of [7-¹⁴C]phenylglyoxal revealed that the loss of ICDH activity was linearly related to the incorporation of phenylglyoxal into the enzyme, and that complete inactivation corresponded to a reaction stoichiometry of approximately one per subunit. In contrast, incubation of phosphorylated ICDH with phenylglyoxal gave no detectable incorporation of phenylglyoxal into the enzyme [15]. These results suggest strongly that the active site of ICDH contains one particularly reactive arginine residue which can be protected against modification either by binding of NADP⁺ or by phosphorylation. The amino acid sequence round the site of phosphorylation of ICDH [16, 17], and indeed the complete sequence of the protein [18], are already known. It is intriguing to note that there is an arginine residue immediately adjacent to the phosphorylatable serine residue, on its N-terminal side. Could it be this arginine residue that is susceptible to phenylglyoxal? Unfortunately, the arginine–phenylglyoxal adduct in ICDH proved to be unstable over the time and under the conditions required for generation of a radioactively labelled peptide and its isolation by h.p.l.c. As a consequence, we have been unable to answer this question directly. We therefore digested ICDH that had been either ³²P-phosphorylated or incubated with [⁷-¹⁴C]phenylglyoxal rapidly with thermolysin, separated the resulting peptides by SDS/polyacrylamide-gel electrophoresis and dried the gels immediately for autoradiography. This experiment showed that the same peptide was labelled in both cases. This is good, albeit indirect, evidence that the arginine residue at the active site of ICDH is immediately adjacent to the phosphorylatable serine residue.

Thorsness & Koshland [18] have recently investigated the mechanism underlying the effects of phosphorylation on ICDH by using site-directed mutagenesis. They showed that a mutant in which the phosphorylatable serine residue had been replaced by an aspartate residue was completely inactive. This is, of course, exactly the result that would be predicted from our hypothesis. Unfortunately, Thorsness & Koshland [18] did not show that the mutant ICDH folded correctly. Until this has been done, it cannot be concluded that the inactivation caused by this mutation resulted from the introduction of a negative charge rather than from some gross effect on the conformation of the enzyme. Nevertheless, site-directed mutagenesis will provide a powerful tool for future analysis of the effects of phosphorylation on the conformation and activity of ICDH.

The location of the phosphorylation site of ICDH close to its active site raises questions concerning the recognition of ICDH by the kinase/phosphatase. Most eukaryotic protein kinases are able to phosphorylate effectively quite small peptides that correspond in sequence to the sites of phosphorylation in intact proteins. In these cases, recognition of elements of primary structure plays some part in the overall process of enzyme/substrate recognition. We therefore synthesized a 14-residue peptide corresponding to the region round the phosphorylation site in ICDH [16]. ICDH kinase was unable to phosphorylate this peptide to a detectable extent. This result suggests that the kinase recognizes some large part of the native ICDH molecule rather than a small linear sequence of amino acid residues.

While there is much information available about the effects of phosphorylation on the biological activities of many proteins, there is very little understanding of the underlying effects on protein conformation. The phosphorylation of ICDH presents a very good model system in which the effects of phosphorylation on protein conformation can be studied. We have already developed a conceptual framework to explain how phosphorylation affects ICDH. Moreover, the relatively small size of ICDH makes it amenable to high-resolution structural studies, its kinetic properties are well understood and the availability of the cloned gene for ICDH makes site-directed mutagenesis possible. These techniques will be used to refine and extend our current understanding of the structure and regulation of ICDH.

Glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides

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Glucose-6-phosphate dehydrogenase (G6PD) from Leuconostoc mesenteroides is one of a small number of dehydrogenases that can utilize either NAD or NADP. The physiological roles of these two coenzymes are quite different, and the enzymes that utilize them are generally specific for one or the other coenzyme. An essential function of G6PDs in animals, for example, is to generate NADPH for reductive biosyntheses. Although these enzymes can utilize NAD⁺, they do so only at non-physiological NAD⁺ concentrations [1, 2]. Presumably the coenzyme-binding sites of dehydrogenases have evolved so that they can distinguish between NAD and NADP. Leuconostoc mesenteroides is a facultatively anaerobic micro-organism which metabolizes glucose to generate lactic acid, ethanol and CO₂ [3]. The NADH generated in the reaction catalysed by G6PD is utilized in the reductive steps of this heterolactic fermentation; when NADPH is produced by G6PD it is used for the biosynthesis of fatty acids [4]. Thus, G6PD plays an essential, amphibolic role in the metabolism of L. mesenteroides.

Two questions that have motivated our studies of L. mesenteroides G6PD are: what protein structural features enable this enzyme to bind both NAD⁺ and NADP⁺ to the same site? and how are the catalytic activities with NAD⁺ and NADP⁺ different? L. mesenteroides G6PD is a relatively simple protein consisting of two identical subunits of molecular mass 54 800 Da, with no propensity to form larger aggregates [5, 6]. Kinetic studies revealed that the enzyme obeys Michaelis–Menten kinetics with no evidence of cooperative kinetics.

The Kₘ for NAD⁺ is nearly 20 times greater than the Kₘ for NADP⁺, whereas kₐ for the NAD-linked reaction is nearly twice kₐ for the reaction with NADP⁺ (Table 1) [7, 8]. Kinetic and binding studies showed that NAD⁺ and NADP⁺ bind to the same site on the enzyme [7, 9], although it is clear that their modes of binding differ. The steady-state kinetic mechanisms of the NAD- and NADP-linked reactions differ. When NADP⁺ is the coenzyme, the mechanism is ordered sequential, with coenzyme binding first; the NADP-linked reaction proceeds via a random-order mechanism [10]. This difference appears to be an essential factor underlying the enzyme’s ability to regulate the utilization of NAD⁺ or NADP⁺. Our initial attempts to find metabolic intermediates or other ligands that could affect the NAD- and NADP-linked reactions differentially were unsuccessful. We therefore devised an assay method that enabled us to measure the rates of both the NAD-linked and the NADP-linked reaction simultaneously. This technique, the dual absorbance assay [8], makes use of the fact that the thionicotinamide analogues of the reduced coenzymes, S-NADH and S-NADPH, absorb maximally at 400 nm, and of the high reactivity of S-NAD⁺ and S-NADP⁺ with L. mesenteroides G6PD. The Kₘ values for the thionicotinamide analogues are substantially lower than, and kₐ/Kₘ values are comparable with, those for the natural coenzymes (Table 1). Using mixtures of NAD⁺ and S-NADP⁺, or NADP⁺ and S-NAD⁺, and measuring the simultaneous increase in absorbance at 340 nm and 400 nm, we measured both the NAD- and NADP-linked reactions simultaneously. Using this technique, we found that high concentrations of glucose 6-phosphate and high concentration ratios of NADPH to NADP⁺ inhibit the NADP-linked, but stimulate the NAD-linked reaction [8, 11]. It must be emphasized that these differential effects on the two activities are only evident when both reactions are proceeding simultaneously, as they depend on the difference in kinetic mechanisms for the two reactions. Although we have no evidence that G6PD is regulated in this manner in vivo, the effects of glucose 6-phosphate and NADPH to NADP⁺ ratio on the NAD- and NADP-linked reactions are consistent with the roles of the two reactions in the metabolism of L. mesenteroides [8, 10, 11].

Although the kₐ/Kₘ values for NAD⁺ and NADP⁺ differ only 10-fold, the Kₐ values for the two coenzymes differ approximately 1000-fold (Table 1). Dissociation constants for both coenzymes have been measured by quenching of protein fluorescence [9, 12] and from protection against thermolysin inactivation [13]; the Kₐ for NADP⁺ was also determined by equilibrium dialysis [12]. From these Kₐ values one can calculate that the apparent binding energy of the extra phosphate group of NADP⁺ is 4.16 kcal/mol at

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Table 1. Kinetic and binding constants for coenzymes and their thionicotinamide analogues

Data are from Levy & Daouk [8] and Haghighi & Levy [12]. ND, Not determined.

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