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RESEARCH ARTICLE

Control of D-octopine formation in scallop adductor muscle as revealed through thermodynamic studies of octopine dehydrogenase

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SUMMARY

Octopine dehydrogenase (OcDH) from the adductor muscle of the great scallop, *Pecten maximus* (Linné, 1758), catalyses the NADH-dependent condensation of L-arginine and pyruvate to D-octopine, NAD^+ and water during escape swimming and subsequent recovery. During exercise, ATP is mainly provided by the transphosphorylation of phospho-L-arginine and to some extent by anaerobic glycolysis. NADH resulting from the glycolytic oxidation of 3-phosphoglyceraldehyde to 1,3-bisphosphoglycerate is reoxidized during D-octopine formation. In some scallops D-octopine starts to accumulate during prolonged, strong muscular work, whereas in other species D-octopine formation commences towards the end of swimming and continues to rise during subsequent recovery. The activity of OcDH is regulated by a mandatory, consecutive mode of substrate binding in the order NADH, L-arginine and pyruvate, as demonstrated by isothermal titration calorimetry. The first regulatory step in the forward reaction comprises the binding of NADH to OcDH with a dissociation constant K_d of 0.014±0.006 mmol l^{-1}, which reflects a high affinity and tight association of the apoenzyme with the co-substrate. In the reverse direction, NAD^+ binds first with a K_d of 0.20±0.004 mmol l^{-1} followed by D-octopine. The binary OcDH–NADH complex associates with L-arginine with a K_d of 5.5±0.05 mmol l^{-1}. Only this ternary complex combines with pyruvate, with an estimated K_d of approximately 0.8 mmol l^{-1} as deduced from pyruvate concentrations determined in the muscle of exhausted scallops. At tissue concentrations of pyruvate between 0.5 and 1.2 mmol l^{-1} in the valve adductor muscle of fatigued *P. maximus*, binding of pyruvate to OcDH plays the most decisive role in initiating OcDH activity and, therefore, in controlling the onset of D-octopine formation.

Key words: octopine dehydrogenase, isothermal titration calorimetry, thermodynamic constants, substrate binding order, regulation of D-octopine formation.

INTRODUCTION

Several motile species of molluscs are known and their performance during flight and fight reactions have been of interest for many years. It was von Buddenbrock (von Buddenbrock, 1911), and later Thomas and Gruffydd (Thomas and Gruffydd, 1971) as well as Feder (Feder, 1972) who described the escape reactions of some of the most vivid species. In particular, members of the Pectinidae show spectacular swimming movements that result from water jets produced by strong and repeated valve clappings. This performance is powered by contractions of the phasic part of the prominent adductor muscle located in the middle of the valve. Exploiting this behaviour, the great scallop *Pecten maximus* can avoid or even separate itself from predators, which are usually different species of starfish.

The ATP required for the strenuous escape activity is regenerated in the muscle tissue of the scallop from the transphosphorylation of phospho-L-arginine, which also results in an increase of L-arginine. When the phosphagen pool is almost depleted, anaerobic glycolysis starts to deliver ATP with a concomitant increase of NADH and pyruvate. Immediate oxidation of NADH to NAD^+, necessary for a continuous, glycolytic ATP supply, is catalysed by octopine dehydrogenase (OcDH; EC 1.5.1.11, N^2-(D-1-carboxyethyl)-L-arginine:NAD^+-oxidoreductase), the terminal enzyme of anaerobic glycolysis in many molluscs. This enzyme, which was initially reported and characterized from the adductor muscle of *P. maximus* (van Thoai and Robin, 1959; van Thoai et al., 1969), catalyses the following reaction:

$$\text{NADH} + \text{H}^+ + \text{L-arginine} + \text{pyruvate} \rightleftarrows \text{D-octopine} + \text{NAD}^+ + \text{H}_2\text{O} .$$

Thus, OcDH, like lactate dehydrogenase, maintains the cytoplasmic redox balance to some extent in anaerobically working muscles (Gäde and Grieshaber, 1986). The product D-octopine accumulates in the tissue, as demonstrated in the working mantle muscle of the squid *Loligo vulgaris* (Grieshaber and Gäde, 1976), in the spadix of the chambered nautilus, *Nautilus pompilius* (Hochachka et al., 1977), and in several other invertebrate species (Grieshaber et al., 1994).

Details of D-octopine formation and accumulation have been particularly studied in various species of vigorously swimming Pectinidae. In some species, such as *Pecten jacobaeus* (Grieshaber and Gäde, 1977), *Placopecten magellanicus* (de Zwaan et al., 1980; Livingstone et al., 1981) and *Argopecten irradians concentricus* (Chih and Ellington, 1983; Chih and Ellington, 1986), the D-octopine content increases during escape swimming or during electrical stimulation as, for example, in the adductor muscle of *Pecten alba* (Baldwin and Opie, 1978) and continues to accumulate to some extent during early recovery. In other bivalves, such as *P. maximus* or *Chlamys opercularis*, D-octopine synthesis commences towards the end of swimming, but preferentially accumulates in the muscle during recovery (Gäde et al., 1978; Grieshaber, 1978). Presumably,

OcDH activity during swimming and recovery is controlled by a time-dependent increase in the cytoplasmic concentration of substrates as well as by a consecutive or random order of coenzyme and substrate binding to the apoenzyme (Monneuse-Doublet et al., 1978; Schrimsher and Taylor, 1984; Chih and Ellington, 1986).

During the last 50 years the kinetic mechanisms of OcDH have been investigated by several authors from different laboratories. Differential spectroscopy and fluorometry were used to describe steady-state kinetics (Luisi et al., 1973; Doublet and Olomucki, 1975; Monneuse-Doublet et al., 1978; Chih and Ellington, 1983; Chih and Ellington, 1986) and the formation of enzyme–substrate complexes (Doublet and Olomucki, 1975; Olomucki et al., 1975; Schrimsher and Taylor, 1984). Stopped-flow as well as isotope replacement methods were applied to gain some insight into the rate-limiting steps for enzyme turnover (Doublet et al., 1975).

From these data, Doublet and Olomucki (Doublet and Olomucki, 1975) and Monneuse-Doublet et al. (Monneuse-Doublet et al., 1978) postulated that the catalytic mechanism of OcDH from the adductor muscle of *P. maximus* obeys a bi-ter sequential mechanism, in which NADH associates first with the enzyme in the forward direction, followed by the consecutive binding of L-arginine and pyruvate. Schrimsher and Taylor (Schrimsher and Taylor, 1984) also reported the primary binding of NADH to OcDH, but in contrast proposed a final complex formation with L-arginine and pyruvate binding in a random order. In the direction of D-octopine oxidation, NAD^+ was assumed to associate with the enzyme prior to D-octopine and the products then released in the consecutive order of pyruvate, L-arginine and NADH (Doublet et al., 1975) or in a random process (Schrimsher and Taylor, 1984). Furthermore, so-called 'dead-end complexes' were reported, which supposedly include an OcDH–NAD^+–L-arginine–pyruvate complex and an OcDH–NADH–D-octopine complex (Doublet et al., 1975).

The decline of the NAD^+/NADH ratio as well as elevations in metabolite concentrations and H^+ during the course of burst contractions have been reported to regulate D-octopine formation *in vivo* in scallops. In particular, the increase of pyruvate concentration is assumed to activate OcDH catalysis in the direction of D-octopine synthesis (Walsh et al., 1984; Chih and Ellington, 1986; Bailey et al., 2003). In addition, dead-end complexes are inhibited by their substrates (NADH, pyruvate) and by their products (NAD^+ and octopine), which could add some fine tuning to the enzyme activity (Doublet et al., 1975; Schrimsher and Taylor, 1984).

Heterologously expressed OcDH from the valve adductor muscle of the great scallop *P. maximus* provided sufficient amounts of homogeneous protein (Müller et al., 2007) to allow for X-ray analysis of the crystal structure of OcDH in complex with NADH, as well as with the ternary complexes NADH–L-arginine and NADH–pyruvate (Smits et al., 2008). Detailed information about the principles of substrate recognition, ligand binding and the reaction mechanism could thus be achieved on a molecular level. Furthermore, information derived from these crystal structures and from solution NMR (Smits et al., 2010) proved the sequence of ligand binding in the order of NADH, L-arginine and pyruvate.

Despite this wealth of information available on the physiology and biochemistry of OcDH, thermodynamic details of the various interactions between enzyme and substrates are still missing. To extend our knowledge of the cellular function of this enzyme and its regulation, we used isothermal titration calorimetry (ITC) (Leavitt and Freire, 2001) and analysed the binding energetics of the interaction of OcDH with the reduced as well as the oxidized coenzyme and the different substrates. We determined the Gibbs free energies, enthalpy and entropy values, as well as the dissociation constants between OcDH and its substrates. The data obtained from this study were used to further support the mandatory sequence of ligand binding to OcDH and to deduce the main regulatory steps of the enzyme's activity during muscular work and subsequent recovery.

MATERIALS AND METHODS

Materials

All chemicals were of analytical grade and were used without further purification. NADH and NAD^+ were obtained from Roche (Mannheim, Germany), pyruvate and L-arginine from Sigma (München, Germany). D-Octopine was prepared as described by elsewhere (Tempé, 1983) and its purity was tested by thin-layer chromatography as well as by an optical enzymatic assay (Grieshaber et al., 1978). Buffer substances were purchased from AppliChem (Darmstadt, Germany) or Riedel de Haen (Seelze, Germany).

Expression and purification

OcDH was heterologously expressed in *Escherichia coli* strain ER2566 and purified to homogeneity as described previously (Müller et al., 2007).

Enzyme assay

Enzyme activities were determined by following the decrease in absorbance of NADH at 340 nm using an Uvikon 810 spectrophotometer (BioTek, Neufahrn, Germany) or a Pharmacia Ultrospek 200-photometer (GE Healthcare, Freiburg, Germany) at a temperature of 25°C. The reaction mixtures contained 50 mmol l^{-1} triethanolamine hydrochloride (TRA), pH 7.5, 3.0 mmol l^{-1} pyruvate, 0.16 mmol l^{-1} NADH and a selected volume of enzyme solution. The reaction was started by adding 5.5 mmol l^{-1} L-arginine.

Isothermal titration calorimetry

ITC experiments were performed using a VP-ITC titration calorimeter system (Microcal Inc., GE Healthcare). OcDH solutions were prepared by dialysis of an aliquot of OcDH against 100 mmol l^{-1} Hepes, pH 7.0 at 4°C. Ligand solutions were prepared in the same buffer. All solutions were degassed for 10 min with gentle stirring under vacuum. Solutions of OcDH (0.15–0.5 mmol l^{-1}) were added to the sample cell (1.4 ml volume) and titrated with various substrate concentrations (15–150 mmol l^{-1}). A typical experiment consisted of 25 injections of 10 µl and 20 s duration each. The injection syringe was rotated at 310 r.p.m. and the time interval between the injections was 240 s. Enthalpy changes ($\Delta_r H^0$) due to protein dilution were determined by titrating buffer into the protein solution and were found to be negligible. All experiments were conducted at 298 K. The heat of ligand dilution was determined by injection of ligand solution into the buffer. Three independent measurements were used to estimate the mean values of the dissociation constants (K_d) and thermodynamic parameters of NADH and NAD^+ binding to OcDH at 298 K and pH 7.0. Raw data were collected, corrected for heats of dilution of ligand injections, and integrated using MicroCal Origin 7.0 software. A single-site binding model was fitted to the data by non-linear regression analysis to yield K_d values and $\Delta_r H^0$ of the specific reaction. The two parameters were used to calculate changes of free energy and entropy of the specific reaction according to Eqn 1:

$$\Delta_r G^0 = -RT\ln K_d = \Delta_r H^0 - T\Delta_r S^0 , \quad (1)$$

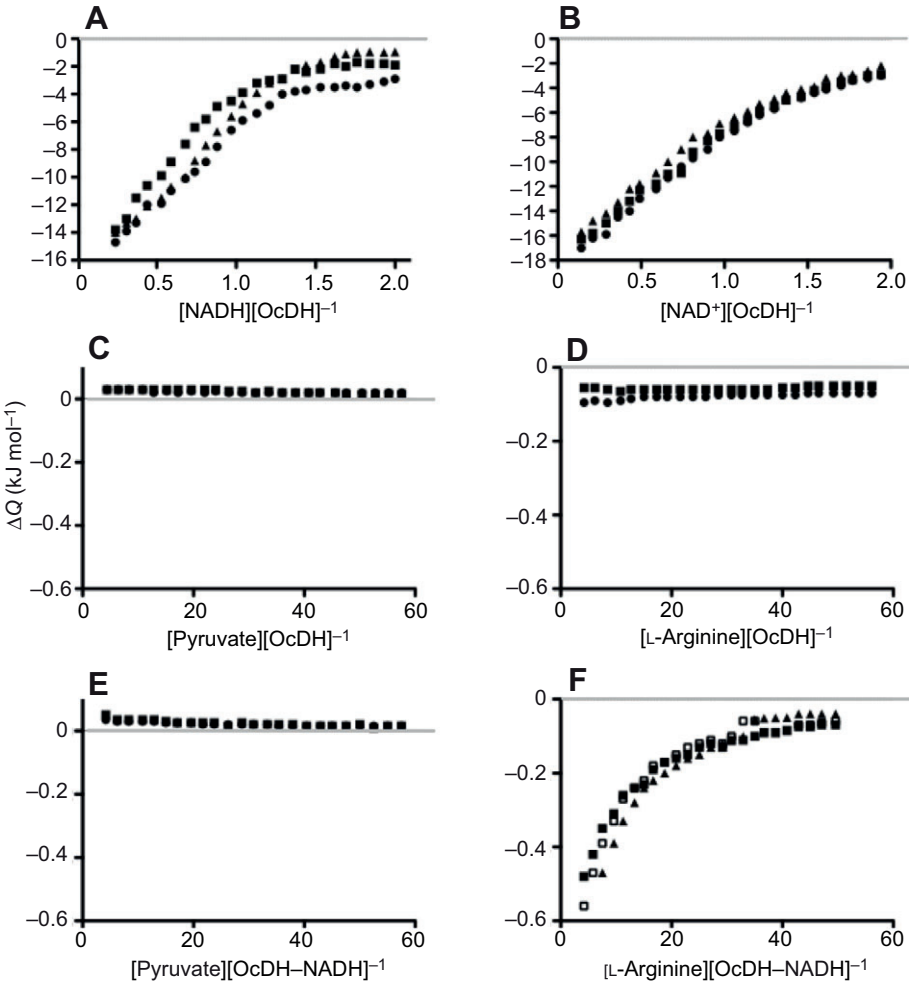

Fig. 1. NADH, NAD^+, pyruvate and L-arginine binding to the octopine dehydrogenase (OcDH) apoenzyme and holoenzyme. (A) 1.75 mmol l^{-1} NADH was titrated into 0.175 mmol l^{-1} OcDH solution. (B) 5 mmol l^{-1} NAD^+ was titrated into 0.5 mmol l^{-1} OcDH solution. (C) 150 mmol l^{-1} pyruvate was titrated into 0.5 mmol l^{-1} OcDH. (D) 150 mmol l^{-1} L-arginine was titrated into 0.5 mmol l^{-1} OcDH. (E) 150 mmol l^{-1} pyruvate was titrated into 0.5 mmol l^{-1} OcDH in the presence of 2.5 mmol l^{-1} NADH. (F) L-Arginine binding isotherms were determined by titrating 75 mmol l^{-1} L-arginine into 0.3 mmol l^{-1} OcDH in the presence of 1.5 mmol l^{-1} NADH. Titrations were performed in 100 mmol l^{-1} Hepes, pH 7.0, 25°C. Binding isotherms were obtained by integration and normalization of the raw data and by correction for the heat of ligand dilution. For the purpose of simplification, only one representative result of double (when no binding occurs) and triplicate measurements is shown.

where $\Delta_r G^0$ is the standard Gibbs free energy of the reaction, R is the universal gas constant, T is the absolute temperature, $\Delta_r H^0$ is the calorimetrically determined enthalpy of reaction, and $T\Delta_r S^0$ is the standard entropy of the reaction at 298 K. During the fitting routine the number of binding sites, n, was fixed to 1, with the exception of the coenzyme titrations. This procedure allows for the accurate thermodynamic information from ITC experiments in which the c value (where c is the product of the macromolecule concentration, the number of binding sites and the binding constant K_a) is less than 10. Assuming that the number of binding sites is known, a sufficient part of the binding isotherm can be used for analysis, if the concentrations of macromolecule and ligand are precisely known and if there is an adequate level of signal-to-noise ratio (Turnbull and Daranas, 2003).

RESULTS

Coenzyme binding

As it was evident from previous work (Luisi et al., 1975) that changes of K_d and the Michaelis–Menten constant (K_m) for the coenzyme varied only around 10% with temperatures between 25 and 30°C, all measurements were performed at 25°C. In Fig. 1A,B, the integrated heat $\Delta_r H^0$ resulting from single injections of coenzymes into the OcDH apoenzyme solution is plotted against the molar ratio of coenzyme to enzyme. NADH or NAD^+ bind first, with a binding

Table 1. Dissociation constants and thermodynamic parameters for binding of co-substrates and substrates to octopine dehydrogenase from the adductor muscle of *Pecten maximus*

Experiment	K_d (mmol l^{-1})	ΔG^0 (kJ mol^{-1})	ΔH^0 (kJ mol^{-1})	$T\Delta S^0$ (kJ mol^{-1})
NADH in OcDH	0.014±0.006	–27.5±1.2	–15.0±1.4	+12.4 ±2.6
NAD^+ in OcDH	0.18±0.046	–21.3±0.1	–24.7±0.7	–3.3±0.7
L-Arginine in OcDH–NADH	5.52±0.67	–12.9±0.3	–12.5±1.2	+0.3±1.4
D-Octopine in OcDH–NADH	0.8±0.1	–17.7±0.3	–9.0±1.4	+8.7±1.7
Pyruvate in OcDH–NAD^+–L-arginine	3.55±0.31	–14.0±0.3	–38.1±2.7	–24.1± 3.01
Arginine in OcDH–NAD^+–pyruvate	3.2±0.7	–14.7±0.3	–29.7±1.9	–15.0±1.6
L-Arginine + pyruvate in OcDH–NAD^+	3.1±1.0	–14.3±0.4	–47.5±4.9	–33.5±5.3

OcDH, octopine dehydrogenase.
K_d, dissociation constant; ΔG^0, standard Gibbs free energy; ΔH^0, standard enthalpy; $T\Delta S^0$, standard entropy.

stoichiometry n of 0.8±0.07 and 0.9±0.04 ligand mol^{-1} monomeric OcDH, respectively. The binding of NADH results in an exothermic Δ_rH^0 of –15.0±1.4 kJ mol^{-1}, whereas complex formation with NAD$^+$ shows a significantly higher enthalpy contribution of –24.7±0.7 kJ mol^{-1} (Table 1). The enthalpic energy contribution of NADH binding is comparatively low, but is still in contrast to the results of Luisi et al. (Luisi et al., 1975) who found a Δ_rH^0 of 0. A non-thermic entropy-driven binding of NADH can also be found in horse liver ethanol dehydrogenase (Hinz, 1983).

The dissociation constant K_d (K_d=1/K_a) for NADH and NAD$^+$ was estimated to be 0.014±0.006 and 0.18±0.046 mmol l^{-1}, respectively (Table 1). These values are in good agreement with dissociation constants from *P. maximus* OcDH derived from fluorometric studies by Luisi et al. (Luisi et al., 1975), who measured K_d constants of 0.02 and 0.38 mmol l^{-1} for NADH and NAD$^+$, respectively. NMR spectroscopy provided K_d values for NADH of 0.06 and 0.40 mmol l^{-1} for NAD$^+$ (Smits et al., 2010). For various NAD$^+$/NADH dehydrogenases isolated from different vertebrate tissues, similar dissociation constants were obtained (Subramanian and Ross, 1978; Hinz, 1983).

A positive entropic contribution $T\Delta_rS^0$ of +12.4±2.6 kJ mol^{-1} characterizes the formation of the OcDH–NADH complex and is in contrast to the binding of NAD$^+$ with OcDH with a negative entropy $T\Delta_rS^0$ of –3.3±0.7 kJ mol^{-1} (Table 1). Previously, Luisi et al. (Luisi et al., 1975) deduced a large and positive entropy change from the temperature-independent binding of NADH. The authors calculated a value of Δ_rS^0 of +26.9 kJ mol^{-1} at a temperature of 300 K, which coincides fairly well with our data derived from ITC assays in which Hepes was used instead of a phosphate buffer system. A positive entropy value of +17.9 kJ mol^{-1} of NADH binding has also been reported for NAD-dependent alcohol dehydrogenase from horse liver (Subramanian and Ross, 1978; Hinz, 1983).

The value of Gibbs free energy of Δ_rG^0 of –27.5±1.2 kJ mol^{-1} for NADH binding to OcDH corresponds well with a mean Δ_rG^0 of –31±6 kJ mol^{-1} calculated from data obtained for alcohol and lactate dehydrogenase. The free energy of OcDH–NAD$^+$ complex formation resulted in a value of Δ_rG^0 of –21.3±0.1 kJ mol^{-1} (Table 1), which is close to the mean Δ_rG^0 of –18±4 kJ mol^{-1} reported for lactate and malate dehydrogenase (Hinz, 1983).

In general, the thermodynamic data of NADH/NAD$^+$ binding to OcDH obtained by ICT compare well with those reported earlier by researchers using indirect methods (Baici et al., 1974; Doublet and Olomucki, 1975). They are also in line with studies performed on other dehydrogenases (Hinz, 1983). However, complex formation of OcDH–NADH differs by having a large positive entropic energy gain, which is similar to alcohol dehydrogenase from horse liver (Subramanian, 1979).

As previously proposed (Luisi et al., 1975), the binding of NADH to OcDH introduces a conformational change in OcDH, which can be deduced from the shifting of several amide cross-peaks seen in the NMR spectra (Smits et al., 2010). The results also corroborate the three-dimensional structure obtained by X-ray crystallography of the OcDH–NADH complex, suggesting a new and stabilized conformation of the protein following binding of the coenzyme (Smits et al., 2008). This stable protein conformation creates the binding site for the second substrate, L-arginine.

Binding of L-arginine and pyruvate to OcDH–NADH

When either L-arginine or pyruvate was added to the apoenzyme, only small heat signals were obtained, corresponding to non-specific heat effects (Fig. 1C,D). In accordance with previous findings (Monneuse-Doublet et al., 1978; Smits et al., 2010), specific pyruvate or L-arginine binding to the apoenzyme could not be detected, even in the presence of high substrate concentrations as proposed by Schrimsher and Taylor (Schrimsher and Taylor, 1984). When pyruvate was titrated into OcDH–NADH, again no binding was found (Fig. 1E). These results also confirm data derived from NMR spectra that show no pyruvate-dependent shifting of cross-peaks (Smits et al., 2010). Obviously, pyruvate cannot associate with OcDH–NADH when L-arginine is absent from the complex.

L-Arginine bound to OcDH–NADH (Fig. 1F) resulting in a K_d of 5.52±0.67 mmol l^{-1}, an enthalpy change Δ_rH^0 of –12.5±1.2 kJ mol^{-1} and a small entropy gain $T\Delta_rS^0$ of +0.3±1.4 kJ mol^{-1} (Table 1). An identical K_d value of 5.5±0.05 mmol l^{-1} for L-arginine has been derived from NMR studies (Smits et al., 2010). The reaction proceeds with an exergonic Δ_rG^0 of –12.9±0.3 kJ mol^{-1} (Table 1). The L-arginine-induced conformational change obviously generates the binding site for pyruvate and allows the α-ketoacid to be located in close proximity to NADH (Smits et al., 2010). Unfortunately, the binding constant for the binding of pyruvate to the preformed OcDH–NADH–L-arginine complex cannot be estimated by ITC because the change in free energy developing during the formation of D-octopine would conceal the heat dissipation resulting from the binding process. Nevertheless, it can be confirmed by calorimetry that D-octopine formation follows a bi-ter binding mechanism in the order of NADH, L-arginine and pyruvate as proposed earlier (Luisi et al., 1975) and also suggested by analysis of the three-dimensional structure of OcDH (Smits et al., 2008).

D-Octopine binding and dead-end complex formation

Similar to L-arginine and pyruvate, D-octopine does not bind to the apoenzyme (Fig. 2A). This result is in contrast to the work of Schrimsher and Taylor (Schrimsher and Taylor, 1984), who postulated the formation of an OcDH–D-octopine complex. Selective measurements of D-octopine binding to OcDH–NAD$^+$ are again unsuitable as a result of the heat dissipation occurring during D-octopine oxidation to pyruvate, L-arginine and NADH.

Binding of D-octopine to the OcDH–NADH complex (Fig. 2B) is, however, possible. Here, a dissociation constant K_d of 0.8±0.1 mmol l^{-1}, an exothermic heat dissipation Δ_rH^0 of –9.0±1.4 kJ mol^{-1} and an entropic energy gain $T\Delta_rS^0$ of +8.7±1.7 kJ mol^{-1} were determined, which amount to a free energy change Δ_rG^0 of –17.7±0.3 kJ mol^{-1} (Table 1). These data confirm the formation of a ternary complex between the first substrate of the forward reaction (NADH) and its product (D-octopine), termed a dead-end complex (Olomucki et al., 1975; Monneuse-Doublet et al., 1978). A ternary complex similar to this OcDH–NADH–D-octopine complex in which an enzyme associates with a coenzyme and a substrate, both of which are in the same oxidation state has been described for lactate dehydrogenase and was called an ‘abortive complex’ (Holbrook and Gutfreund, 1973).

Another dead-end complex could possibly result from the complex OcDH–NAD$^+$ together with L-arginine or pyruvate. In accordance with the findings of Monneuse-Doublet et al. (Monneuse-Doublet et al., 1978), binding of either substrate to the OcDH apoenzyme could not be detected (Fig. 2C,D), even at high substrate concentrations, as supposed by Schrimsher and Taylor (Schrimsher and Taylor, 1984). However, if L-arginine together with the OcDH–NAD$^+$ complex was present in the reaction mixture prior to the addition of pyruvate, the titration of the α-ketoacid resulted in a K_d of 3.55±0.31 mmol l^{-1}. A negative enthalpic contribution of Δ_rH^0 of –38.1±2.7 kJ mol^{-1} and a decrease in $T\Delta_rS^0$ of

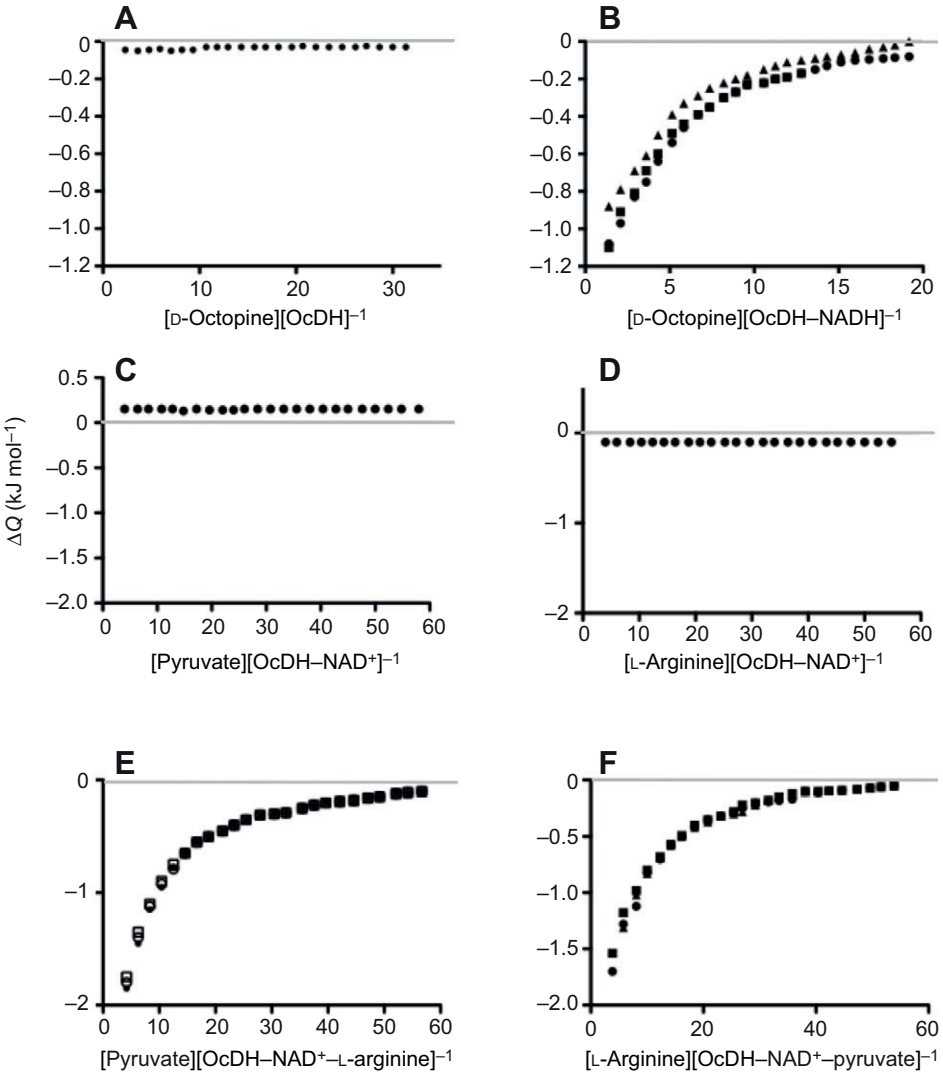

Fig. 2. D-Octopine binding to OcDH, as well as D-octopine, pyruvate and L-arginine binding to the complementary OcDH–NADH complex, and binding of pyruvate and L-arginine to the OcDH–NAD^+–L-arginine complex or the OcDH–NAD^+–pyruvate complex, respectively. (A) 50 mmol l^{-1} D-octopine was titrated into 3.0 mmol l^{-1} OcDH. (B) 15 mmol l^{-1} D-octopine was titrated into 0.15 mmol l^{-1} OcDH–NADH complex. (C) 150 mmol l^{-1}pyruvate was titrated into 0.46 mmol l^{-1} OcDH–NAD^+ complex. (D) 75 mmol l^{-1} L-arginine was titrated into 0.46 mmol l^{-1} OcDH–NAD^+ complex. (E) 75 mmol l^{-1} pyruvate was titrated into a 0.26 mmol l^{-1} OcDH–NAD^+ complex containing 1.5 mmol l^{-1} L-arginine. (F) 75 mmol l^{-1} L-arginine was titrated into 0.26 mmol l^{-1} OcDH–NAD^+ complex containing 1.5 mmol l^{-1}pyruvate. Titrations were performed in 100 mmol l^{-1} Hepes, pH 7.0, 25°C. Binding isotherms were obtained by integration and normalization of the raw data and by correction for the heat of ligand dilution. For the purpose of simplification, only a representative result of double (when no binding occurs) and triplicate measurements is shown.

–24.1±3.01 kJ mol^{-1} amounts to a Δ_rG^0 of –14.0±0.3 kJ mol^{-1} (Fig. 2E; Table 1). *Vice versa*, when L-arginine was added to the OcDH–NAD^+ complex preincubated with pyruvate (Fig. 2F), enthalpic and entropic energy values were also negative. The K_d and Δ_rG^0 values correspond to those obtained for the formation of the OcDH–NAD^+ complex (Table 1). Finally, if L-arginine and pyruvate were added simultaneously to OcDH–NAD^+, a dead-end complex originated with an 'apparent' K_d of 3.1±1.0 mmol l^{-1}, Δ_rH^0 of –47.5±4.9 kJ mol^{-1} and a decrease in $T\Delta_rS^0$ to –33.5±5.3 kJ mol^{-1}, which amounts to a Δ_rG^0 of –14.3±0.4 kJ mol^{-1} (Table 1).

DISCUSSION

Mandatory and ordered sequence of substrate binding

Calorimetrically estimated thermodynamic parameters of substrate binding to OcDH allow for a thorough understanding of the energetics governing enzyme–substrate interactions as well as an interpretation of ligand-binding sequences, and a less speculative insight into the regulatory function of this enzyme. In addition, they supplement knowledge of the kinetic and structural properties of OcDH reported previously (Doublet and Olomucki, 1975; Doublet et al., 1975; Monneuse-Doublet et al., 1978; Smits et al., 2008; Smits et al., 2010).

Complex formation with NADH as a first substrate is a typical feature of dehydrogenases (Hinz, 1983) and it also holds true for OcDH isolated from the adductor muscle of the great scallop *P. maximus*. OcDH from this species binds NADH with a low K_d of 0.014 mmol l^{-1} and a substantial positive entropic gain $T\Delta_rS^0$ of +12.4 kJ mol^{-1} (Table 1). The thermodynamics of the OcDH–NADH binding process are quite unique, in particular with regard to the high entropic value, as most other dehydrogenases show large negative enthalpies and also negative entropies (Hinz, 1983). Thus, the enzyme is geared for tight and selective complex formation as soon as NADH increases, as for example during the onset of anaerobic glycolysis in strenuously working muscles and/or during their subsequent recovery. Chih and Ellington reported a 3.1- to 3.5-fold decline of the cytoplasmic NAD^+/NADH ratio during escape swimming in the adductor muscle of the bay scallop *A. irradians concentricus* (Chih and Ellington, 1986). It can therefore be concluded that an increase of NADH concentration serves as a first and sensitive metabolic step in OcDH activation in *P. maximus*.

Binding of pyruvate to OcDH–NADH prior to L-arginine was never observed. In contrast, binding of L-arginine before pyruvate is essential for further complex formation. The OcDH–NADH–L-arginine complex assembles with a K_d of 5.5 mmol l^{-1} during an enthalpically as well as a small entropically favoured reaction (Table 1). The concentration of L-arginine, which according to Gäde et al. (Gäde et al., 1978) ranges from 24 to 49 mmol l^{-1} in resting and exhausted *P. maximus*, respectively, is more than sufficient for

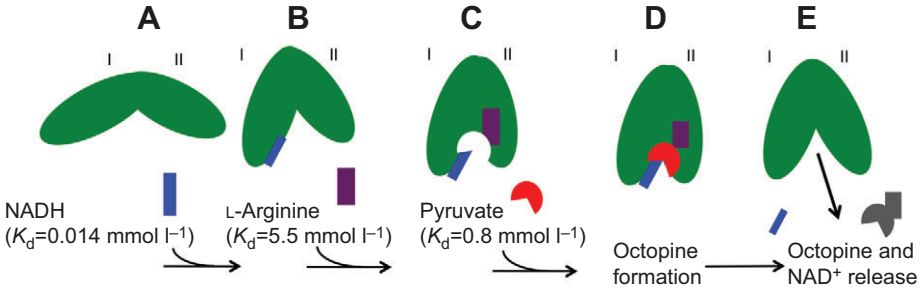

Fig. 3. Schematic drawing of the reaction mechanism of OcDH. (A) The apoenzyme is shown in green, with the two domains, I and II, indicated. (B) NADH (shown in blue) binds to form the OcDH–NADH complex, the conformation of which is stabilized *via* the interaction of NADH with both domains. (C) L-Arginine (shown in purple) binds at domain II and thereby the binding site for the last substrate is created. (D) Pyruvate (shown in red) binds to the OcDH–NADH–L-arginine complex, which then is immediately condensed to D-octopine (E). After the reaction, D-octopine (shown in grey) and NAD$^+$ are released.

complex formation even in resting animals. [In order to understand how substrate changes might influence the binding behaviour to enzymes, substrate levels usually given in μmol g^{-1} wet mass must be converted to concentration in mmol l^{-1} (Chih and Ellington, 1986). Thus, intracellular water needs to be taken into account (Newsholme and Start 1977), which represents approximately 50% of the wet mass of the adductor muscle of *P. maximus* (M.K.G., unpublished observation). Therefore, metabolite levels were converted to concentrations by multiplication by two.] An immediate ternary complex formation with the amino acid is also corroborated by low K_m values for L-arginine ranging from 2.4 to 4.0 mmol l^{-1} depending on co-substrate concentrations (Doublet and Olomucki, 1975; Baldwin and Opie, 1978; Chih and Ellington, 1986).

As no OcDH–NADH complex formation with pyruvate prior to L-arginine binding is possible, the ternary OcDH–NADH–L-arginine complex is poised to react with pyruvate, but pyruvate will only associate with this ternary complex when it is at a high enough concentration. However, cytoplasmic pyruvate concentrations of 0.12 mmol l^{-1} (de Zwaan et al., 1980) and 0.14 mmol l^{-1} (Chih and Ellington, 1986) in resting scallops are obviously too low for complex formation as indicated by the absence of D-octopine in muscle tissue. Only an increase of pyruvate in muscles of scallops during escape swimming or recovery could lead to an OcDH–NADH–L-arginine–pyruvate complex.

An estimate of the dissociation constant K_d for pyruvate

The onset and rate of D-octopine formation can be used to estimate the dissociation constant K_d for pyruvate, which cannot be directly investigated by NMR or ITC because of the immediate energy release during the condensation reaction. Estimated cellular concentrations of pyruvate were reported from *P. magellanicus* (de Zwaan et al., 1980) and *A. irradians concentricus* (Chih and Ellington, 1986). Both species accumulate D-octopine during swimming activity, when pyruvate concentrations were found to increase from 0.12 to 0.52 mmol l^{-1} in the former species and from 0.14 to 1.24 mmol l^{-1} in the latter. Both species start to synthesize D-octopine when the pyruvate concentration is in the range of ~0.6–1.0 mmol l^{-1}. In addition, the apparent K_m values for OcDH (1.5 mmol l^{-1} for pyruvate at pH 7.0 and 25 mmol l^{-1} for L-arginine) (Doublet and Olomucki, 1975) indicate pyruvate concentrations that promote OcDH activity. From these data we propose a K_d value for pyruvate binding of ~0.8 mmol l^{-1}, a concentration that can exert the final regulatory control on D-octopine formation. Thus, the increase in pyruvate concentration during or at the end of escape swimming or during subsequent recovery comprises the second and most decisive regulatory step in activating OcDH catalysis, leading to D-octopine accumulation.

Substrate and product binding to OcDH dead-end complexes

OcDH–NADH–D-octopine and OcDH–NAD$^+$–L-arginine–pyruvate comprise enzyme complexes in which coenzyme and substrates are in the same oxidation state. The assembly of these dead-end complexes controls OcDH activity by substrate and product inhibition, which has been investigated by steady-state kinetics and spectroscopic studies in the presence of different coenzyme and substrate analogues. The majority of these experiments have been performed by two groups, who reported different results concerning the mechanisms of substrate binding and inhibitor binding. These researchers (Baici et al., 1974; Doublet and Olomucki, 1975; Monneuse-Doublet et al., 1978; Olomucki et al., 1975) concluded that D-octopine can only complex with OcDH in the presence of NADH, whereas the binding of pyruvate and L-arginine to the OcDH–NAD$^+$ complex occurs in the order of pyruvate binding before L-arginine. In contrast, Schrimsher and Taylor (Schrimsher and Taylor, 1984), who mainly analysed the nature of substrate inhibition, found that D-octopine can bind directly to the apoenzyme, whereas L-arginine and pyruvate will bind randomly to the OcDH–NAD$^+$ complex.

D-Octopine binding to the apoenzyme could not be substantiated by calorimetric analysis (Fig. 1A). However, in the presence of NADH, D-octopine binding is quite tight (K_d=0.8 mmol l^{-1}) and the formation of the OcDH–NADH–D-octopine complex is enthalpically as well as entropically favoured (Table 1). The D-octopine affinity to the binary complex and can be explained by the similar structure of D-octopine as compared with the transitory intermediate, which is the corresponding Schiff base between pyruvate and L-arginine. The small dissociation constants derived for NADH and D-octopine binding point to a relatively stable ternary complex, which is also likely to exist *in vivo* (Table 1). Comparison of the kinetic data from inhibition studies of OcDH [inhibitor constant K_i values range from 1.5 to 4 mmol l^{-1} D-octopine (Doublet and Olomucki, 1975; Baldwin and Opie, 1978)] with *in vivo* substrate concentrations of D-octopine in exhausted or recovering scallops [9–15 mmol l^{-1} (Baldwin and Opie, 1978; Gäde et al., 1978)] also support the proposal that the OcDH–NADH–D-octopine complex is formed *in vivo* at concentrations of ~12 mmol l^{-1} D-octopine, exerting the known strong product inhibition of OcDH (Doublet and Olomucki, 1975).

Pyruvate (K_d=3.5 mmol l^{-1}) and L-arginine (K_d=3.2 mmol l^{-1}) can only complex with NAD$^+$ if either L-arginine or pyruvate is present in the assay before the second substrate is added. The free energy

$\Delta_r G^0$ is similar for both binding effects and the process is enthalpically favoured. Adding pyruvate and L-arginine simultaneously to the OcDH–NAD^+ complex also results in an enthalpically driven process with an 'apparent K_d of 3.1 mmol l^{-1}' (as two substrates are involved in the binding process, separate constants cannot be assigned to a specific substrate; Table 1). The two substrates are required simultaneously for complex formation and the K_d of the resulting OcDH–NAD^+–L-arginine pyruvate complex reflects a stable bonding with energetics probably similar to those for the formation of the OcDH–NADH–L-arginine–pyruvate complex. However, as the dissociation constant for pyruvate is quite high and pyruvate concentrations are between 0.52 and 1.24 mmol l^{-1} in exhausted scallops (de Zwaan et al., 1980; Chih and Ellington, 1986), D-octopine formation in the forward reaction appears to be substrate inhibited only to a small extent, if at all. Instead, OcDH can function as the terminal step of anaerobic glycolyis until D-octopine reaches concentrations in the range 10–15 mmol l^{-1} during recovery.

Regulation of OcDH activity in swimming and recovering *P. maximus*

In the light of kinetic results from studies on monomeric OcDH from *P. maximus*, mainly published by Anna Olomucki and her coworkers (Doublet and Olomucki, 1975; Doublet et al., 1975), as well as from the thermodynamic results presented here, our understanding of the regulatory behaviour of this enzyme can be extended and used to explain the formation of D-octopine during strong muscular work and subsequent recovery or during recovery only.

OcDH has a high affinity for the reduced coenzyme and will form a tight complex as soon as steady-state concentrations of NADH increase (Fig. 3A,B). The resulting binary complex only can associate with L-arginine. The cellular concentration of this amino acid in resting specimens of *P. maximus* (24.2 mmol l^{-1}) is sufficient to promote the formation of the OcDH–NADH–L-arginine complex, even if there is no activity-related transphosphorylation of phospho-L-arginine to ATP and L-arginine. Therefore, this amino acid substrate cannot exert any significant influence on the regulation of D-octopine formation (Fig. 3C). However, only the OcDH–NADH–L-arginine complex creates the pyruvate-binding site and, therefore, is poised to bind pyruvate when the cellular concentrations of the α-keto acid increase to ~0.8 mmol l^{-1} (Fig. 3C). Thus, regulation of D-octopine formation is controlled by the compulsory order of substrate binding with the formation of a tight, binary OcDH–NADH complex that will immediately bind L-arginine, resulting in the ternary OcDH–NADH–L-arginine complex. In quiescent specimens, such as *A. irradians concentricus*, low steady-state concentrations of pyruvate of 0.13 mmol l^{-1} (Chih and Ellington, 1986) are insufficient to bind to the OcDH–NADH–L-arginine complex and, therefore, no D-octopine will accumulate. D-Octopine synthesis and accumulation in active or recovering scallops will only commence when cellular pyruvate concentrations increase to 0.5–1.2 mmol l^{-1}, which is sufficiently high for the formation of the OcDH–NADH–L-arginine–pyruvate complex (Fig. 3D).

In *P. alba* (Baldwin and Opie, 1978) and *A. concentricus irradians* (Chih and Ellington, 1986), pyruvate levels will rise because of an increase in the anaerobic glycolytic rate at the beginning of escape swimming. Consequently, pyruvate-dependent complex formation will coincide with the onset of strong muscular activity. But in some species, such as *P. maximus* (Gäde et al., 1978), *C. opercularis* (Grieshaber, 1978) and *P. magellanicus* (Livingstone et al., 1981), D-octopine is formed during the first hours of recovery (Fig. 3E). Only then will pyruvate levels transiently start to increase, resulting either from an incomplete aerobic pyruvate oxidation or a total lack of oxygen, and the OcDH–NADH–L-arginine–pyruvate complex will associate giving rise to D-octopine accumulation. But D-octopine synthesis will be attenuated with increasing product concentrations because of the formation of dead-end complexes (Doublet and Olomucki, 1975). When, at the end of recovery, product-dependent complex formation subsides, the OcDH–NAD^+–D-octopine complex will catalyse the reverse reaction and channel pyruvate into an aerobic metabolism. In the light of these biological data, our studies provide the mechanistic framework and the thermodynamic values to understand and explain the function of OcDH *in vivo*.

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