Formation of *N*-hydroxy-*N*-arylacetamides from nitroso aromatic compounds by the mammalian pyruvate dehydrogenase complex

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Bovine, human and porcine heart mitochondria and isolated porcine heart pyruvate dehydrogenase complex (PDHC) pyruvate-dependently form *N*-hydroxy-*N*-arylacetamides from nitroso aromatic compounds, including carcinogenic 4-biphenyl and 2-fluorenyl derivatives. The PDHC-catalysed formation of *N*-hydroxyacetanilide (N-OH-AA) from nitrosobenzene (NOB), through a Ping Pong mechanism, is optimum at pH 6.8 and is accelerated by thiamin pyrophosphate, but is inhibited by thiamin thiazolone pyrophosphate and ATP. K_m^{pyruvate} in the reaction is independent of pH over the range tested, whereas K_m^{NOB} increases at lower pH, owing to ionization of an active-site functional group of pK_a 6.3. The enzymic ionization decreases

$\log (V_{\text{max}}/K_m^{\text{NOB}})$. Isolated pyruvate dehydrogenase (E1), a constitutive enzyme of PDHC, forms N-OH-AA by itself and has comparable kinetic parameters to those of the PDHC-catalysed N-OH-AA formation. The catalytic efficiency of PDHC in the formation of *N*-hydroxy-*N*-arylacetyl amides, due to the steric limitation of the active site of E1, is lowered both by bulky alkyl groups of α -oxo acids and by *p*-substituents (but not an *o*-substituent) on nitrosobenzenes. These nitroso compounds serve as electrophiles in the reaction in which the reductive acetylation step is rate-limiting. The reaction mechanism and other factors affecting *N*-hydroxy-*N*-arylacetyl amide formation are discussed.

INTRODUCTION

N-Hydroxy-*N*-arylacetamides are thought to play important roles as proximate carcinogens in carcinogenicity of *N*-substituted aromatic compounds [1,2]. A well-established pathway for the formation of these proximate carcinogens is cytochrome *P*-450-catalysed *N*-oxidation [2] of *N*-arylacetamides. We have previously reported the mammalian spermatozoon-catalysed formation of *N*-hydroxy-*N*-arylacetamides from nitroso aromatic compounds and pyruvate [3], in which it was shown to be non-oxidative and to be mediated by a mitochondrial enzyme, pyruvate dehydrogenase complex (PDHC). Therefore it is of interest to determine whether the formation of *N*-hydroxy-*N*-arylacetamides from nitroso aromatic compounds proceeds in somatic cells possessing mitochondria other than germ cells. In the present study the ability of mammalian heart mitochondria and isolated porcine heart PDHC to catalyse the formation of *N*-hydroxy-*N*-arylacetamides was evaluated. The experimental evidence obtained in the present investigation shows that all mitochondria tested have the activity and that the biotransformation is mediated by PDHC.

PDHC consists of three major component enzymes: a thiamin pyrophosphate (TPP)-dependent pyruvate dehydrogenase (E1), a dihydrolipoyl transacetylase (E2) with a covalently bound lipoyl moiety, and a flavoprotein dihydrolipoyl dehydrogenase (E3), plus two additional enzymes: a pyruvate dehydrogenase kinase and a pyruvate dehydrogenase phosphatase, which regulate the E1 activity [4–7].

In the PDHC-catalysed formation of *N*-hydroxy-*N*-arylacetamides, transient intermediates derived through E1-catalysed pyruvate decarboxylation [8,9] are thought to be involved as a source of the acetyl moiety. Accordingly, identification of the component enzyme responsible for the PDHC-catalysed for-

mation of *N*-hydroxy-*N*-arylacetamides is of significance in characterizing the reaction. Furthermore, the reaction mechanism and factors affecting the activity, in relation to the abovementioned enzymic acetyl-transfer intermediates, were also investigated.

EXPERIMENTAL

Instrumentation

^1H -n.m.r., m.s. and i.r. spectra were recorded on JEOL JNM-GX270, Hitachi M-2000 and JASCO A-102 spectrometers respectively. Measurements of u.v. spectra and spectrophotometric enzyme assays were carried out on a Shimadzu UV-200S spectrophotometer. H.p.l.c. was performed with a Shimadzu LC-5A liquid chromatograph equipped with an SPD-2A spectrophotometric detector.

Materials

Sodium pyruvate was purchased from Merck. ($\pm$)-Lipoamide, TPP, PDHC (from porcine heart) and phosphotransacetylase (from *Leuconostoc mesenteroides*; EC 2.3.1.8) and sodium salts of α -oxobutyrate, ($\pm$)- β -methyl- α -oxovalerate, α -oxoisovalerate and α -oxoisohexanoate were obtained from Sigma. 2,6-Dichlorophenol-indophenol sodium salt (DCPIP) was obtained from Kishida Chemical Co. (Osaka, Japan). Nitrosobenzene (NOB), obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan), was recrystallized from ethanol before use. Thiamin thiazolone pyrophosphate (TTPP) [10], 2-(1-hydroxyethyl)-TPP [11] and ($\pm$)-dihydrolipoamide [12] were prepared by the cited procedures. *p*-Methyl- and *p*-ethoxymitosobenzenes [13] and the corresponding *N*-hydroxy-*N*-arylacetamides were synthesized according to the reported procedures [14,15]. Other nitroso com-

Abbreviations used: PDHC, pyruvate dehydrogenase complex; E1, pyruvate dehydrogenase; E2, dihydrolipoyl transacetylase; E3, dihydrolipoyl dehydrogenase; TPP, thiamin pyrophosphate; DCPIP, 2,6-dichlorophenol-indophenol sodium salt; TTPP, thiamin thiazolone pyrophosphate; NOB, nitrosobenzene; N-OH-AA, *N*-hydroxyacetanilide; $\text{Me}_2\text{SO}-d_6$, [$^2\text{H}_6$]dimethyl sulphoxide; Me_4Si , tetramethylsilane; EtOH, ethanol; EI, electron impact; CH_3CN , acetonitrile; MeOH, methanol.

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pounds and *N*-hydroxy-*N*-arylacetamides used were synthesized as reported previously [3,16]. *N*-Hydroxy-*N*-phenylacetyl amides (propionyl, butyryl, isobutyryl, isovaleryl and *s*-valeryl) were synthesized from NOB and the corresponding α -oxo acids as described in [16]. Melting points, results of elemental analyses and spectral data of the compounds were as follows.

The *propionyl compound* [17] had m.p. 49–51 °C (white needles from benzene/hexane). Elemental analysis: calc. for $C_9H_{11}NO_2$: C, 65.44; H, 6.71; N, 8.48. Found: C, 65.54; H, 6.70; N, 8.45; 1H -n.m.r. ($[^2H_6]$ dimethyl sulphoxide (Me_2SO-d_6), tetramethylsilane (Me_4Si)) δ (p.p.m.): 1.06 (3H, t, J 7.3 Hz, CH_3), 2.58 (2H, q, J 7.3 Hz, CH_2), 7.10–7.65 (5H, aromatic) and 10.45 (1H, s, OH, exchangeable with 2H_2O); m.s. [electron impact (EI), 40 eV] m/z : 165 (M^+ , the molecular ion), 149, 109 (base peak) and 93; i.r. (KBr) ν_{max} (cm^{-1}): 3150, 1650, 1630, 1480 and 1290; u.v. [ethanol (EtOH)] λ_{max} 254 nm (ϵ 10700).

The *butyryl compound* had m.p. 80–81 °C (white prisms from benzene/hexane) (m.p. 80–81 °C [18]). Analysis: calc. for $C_{10}H_{13}NO_2$: C, 67.02; H, 7.31; N, 7.82. Found: C, 67.12; H, 7.39; N, 7.90; 1H -n.m.r. (Me_2SO-d_6 , Me_4Si) δ (p.p.m.): 0.93 (3H, t, J 7.3 Hz, CH_3), 1.59 (2H, m, J 7.3 Hz, $CH_2CH_2CH_3$), 2.53 (2H, t, J 7.3 Hz, $CH_2CH_2CH_3$), 7.10–7.63 (5H, aromatic) and 10.45 (1H, s, OH, exchangeable with 2H_2O); m.s. (EI, 40 eV) m/z : 179 (M^+), 163 and 93 (base peak); i.r. (KBr) ν_{max} (cm^{-1}): 3150, 1620, 1480 and 1270; u.v. (EtOH) λ_{max} 254 nm (ϵ 10400).

The *isobutyryl compound* had m.p. 53–54 °C (colourless plates from hexane). Analysis: calc. for $C_{10}H_{13}NO_2$: C, 67.02; H, 7.31; N, 7.82. Found: C, 67.15; H, 7.45; N, 7.80; 1H -n.m.r. (Me_2SO-d_6 , Me_4Si) δ (p.p.m.): 1.10 [6H, d, J 7.0 Hz, $CH(CH_3)_2$], 3.18 (1H, m, $CH(CH_3)_2$), 7.12–7.62 (5H, aromatic) and 10.45 (1H, s, OH, exchangeable with 2H_2O); m.s. (EI, 40 eV) m/z : 179 (M^+), 163 and 93 (base peak); i.r. (KBr) ν_{max} (cm^{-1}): 3150, 1620, 1590, 1490 and 1280; u.v. (EtOH) λ_{max} 254 nm (ϵ 10300).

The *isovaleryl compound* had m.p. 69–70 °C (white needles from benzene/hexane). Analysis: calc. for $C_{11}H_{15}NO_2$: C, 68.37; H, 7.82; N, 7.25. Found: C, 68.36; H, 7.90; N, 7.25; 1H -n.m.r. (Me_2SO-d_6 , Me_4Si) δ (p.p.m.): 0.93 [6H, d, J 6.6 Hz, $CH_2CH(CH_3)_2$], 2.12 [1H, m, $CH_2CH(CH_3)_2$], 2.45 [2H, d, J 6.9 Hz, $CH_2CH(CH_3)_2$], 7.14–7.62 (5H, aromatic) and 10.45 (1H, s, OH, exchangeable with 2H_2O); m.s. (EI, 40 eV) m/z : 193 (M^+), 177 and 93 (base peak); i.r. (KBr) ν_{max} (cm^{-1}): 3150, 1620, 1590, 1480 and 1270; u.v. (EtOH) λ_{max} 254 nm (ϵ 10800).

The *s*-valeryl compound was a syrup. Analysis: calc. for $C_{11}H_{15}NO_2$: C, 68.37; H, 7.82; N, 7.25. Found: C, 68.22; H, 7.98; N, 7.09; 1H -n.m.r. (Me_2SO-d_6 , Me_4Si) δ (p.p.m.): 0.87 (3H, t, J 7.3 Hz, CH_2CH_3), 1.08 [3H, d, J 6.6 Hz, $CH(CH_3)_2$], 1.38 [1H, m, $CH(CH_3)_2$], 1.65 [1H, m, $CH(CH_3)_2$], 3.05 [1H, m, $CH(CH_3)_2$], 7.15–7.61 (5H, aromatic) and 10.45 (1H, s, OH, exchangeable with 2H_2O); low- and high-resolution m.s. (EI, 50 eV) m/z : 193 (M^+), 177 and 93 (base peak); calc. for $C_{11}H_{15}NO_2$: 193.1102. Found: 193.1091 (error –1.1 milli-mass units); i.r. (chloroform) ν_{max} (cm^{-1}): 3250, 3000, 1640, 1630, 1590, 1460 and 1210; u.v. (EtOH) λ_{max} 254 nm (ϵ 10100).

m-Chloro-*p*-aminophenol [m.p. 158 °C (decomp.), white needles from benzene] (m.p. 160 °C [19]) was prepared from *N*-hydroxy-*o*-chloroaniline [20] and was acetylated with pyridine/acetic anhydride to give *o*-chloro-*p*-acetoxycetanilide (m.p. 127–128 °C, white needles from benzene/hexane) (m.p. 130 °C [21]). All other chemicals used were of reagent grade.

Preparation of mitochondria

Bovine and porcine hearts, obtained from a local abattoir, were immediately packed in ice. Human hearts, removed from male corpses ages 57 and 68 years at about 22 and 30 h after death

respectively were provided by Dr. T. Takatori (then of the Department of Legal Medicine, School of Medicine, Hokkaido University, Sapporo, Japan, and presently at the Department of Legal Medicine, School of Medicine, Tokyo University, Tokyo, Japan) and stored at –80 °C until use. Mitochondria were isolated from bovine, human and porcine hearts at 0–4 °C as described in [22], except that the tissue was homogenized with a Polytron PT-10 homogenizer (1000 rev./min for 45 s, three times). In the final mitochondrial suspension, glycerol was added to a final concentration of 10% (v/v) and the resultant suspension was stored at –80 °C until use. The typical respiratory-control ratio and the P/O value (with pyruvate and malate) of porcine heart mitochondria, determined by a Clark-type oxygen electrode [23] at 25 °C, were over 2.5 and 2.8 respectively.

Preparation of PDHC

PDHC solution (500 μ l) as supplied was diluted with 25 ml of 100 mM Mops/KOH (pH 6.8) containing 5 mM EDTA, and PDHC was then sedimented by centrifugation at 140000 *g* and 4 °C for 2 h. The PDHC pellet was solubilized in 100 mM Mops/KOH, pH 6.8, followed by brief centrifugation (2000 *g* for 5 min) to remove insoluble material. The supernatant was used for experiments. In this procedure, TPP was almost all removed from PDHC [24], hence the preparation had a TPP requirement for the NADH formation activity (the specific activity was 5–6 units/mg of protein). α -Oxoglutarate and branched-chain- α -oxoacid dehydrogenase activities were not detected in this preparation.

PDHC was resolved into its three major component enzymes [E1 (EC 1.2.4.1), E2 (EC 2.3.1.12) and E3 (EC 1.6.4.3)] as described in [25]. Protein was determined by a modification of the Lowry procedure as described by Markwell et al. [26], with BSA as the standard.

Assay of *N*-hydroxyacetanilide (N-OH-AA) formation activity (N-OH-AA activity)

With mitochondria

N-OH-AA activity was assayed as described previously [3] with a slight modification. The assay medium (final volume 1 ml) consisting of 100 mM Mops/KOH (pH 6.8), 0.5 mM TPP, 10 mM $MgCl_2$, 5 mM NOB [added as 50 μ l of a bis-(2-methoxyethyl) ether solution] and 15 mM-sodium pyruvate was placed in a 2 ml screw-cap vial equipped with a Teflon-faced seal to prevent nitroso-compound volatilization [27]. Incubation was started by adding mitochondrial suspension and proceeded at 30 °C with shaking (60 strokes/min).

Activation energy of N-OH-AA formation, expressed as means $\pm$ S.E., was obtained by the Arrhenius plot of the initial velocities measured over the temperature range 24–37 °C with porcine heart mitochondria (0.24 mg of protein) under the abovementioned conditions. For optimum pH of reaction, the amount of N-OH-AA formed during a 20 min incubation was determined at various pH values (Mes for pH 5.5–6.7 and Mops for pH 6.8–7.5) with porcine heart mitochondria (0.49 mg of protein).

With PDHC and its component enzymes

The assay medium was identical with that described for the mitochondrial N-OH-AA assay. Incubation was started by adding enzyme solution and proceeded at 30 °C without shaking.

In the reaction with 2-(1-hydroxyethyl)-TPP (5 mM), 1 mM NOB was incubated at pH 6.8 for 20 min with and without PDHC (2.9 μ g of protein). In the reaction with 4 mM ($\pm$)-

dihydrolipoamide with 5 mM acetyl-CoA, 1 mM *N*-hydroxy-aniline was incubated at pH 6.8 for 30 min in the presence of PDHC (37 μ g of protein). In the reaction with ($\pm$)-monoacetyl-dihydrolipoamide (2 mM), 1 mM NOB was incubated at pH 6.8 for 10 min in the presence of 0.5 mM TPP, 10 mM MgCl_2 and PDHC (32 μ g of protein).

Since nitroso compounds readily react with thiol compounds [28] and are reduced by NADH [29] in non-enzymic reactions, it was not possible to investigate whether the PDHC-catalysed formation of *N*-hydroxy-*N*-arylacetamides proceeds in the presence of NAD^+ and CoA.

The enzyme assays used are linear with respect to the concentration of protein. To estimate the initial velocity at a given substrate concentration, the amounts of *N*-hydroxy-*N*-arylacetamides formed at three time points were determined by h.p.l.c.

H.p.l.c. analysis

Work-up of the assay mixture for the determination of *N*-hydroxy-*N*-arylacetamides by h.p.l.c. was performed according to the reported procedure [3]. Organic phase-extractable metabolites were chromatographed on a column of LiChrosorb RP-8 (Merck, 4 mm $\times$ 250 mm). The flow rate was 1 ml/min and the eluant, containing 0.01% (w/v) desferal mesylate [30], was acetonitrile (CH_3CN)/water (1:1, v/v), except CH_3CN /methanol (MeOH)/water (1:4:5, by vol.) for *N*-hydroxy-2-chloroacetanilide and CH_3CN /MeOH/water (2:5:3, by vol.) for *N*-hydroxy-4-biphenylacetamide and *N*-hydroxy-2-fluorenylacetamide. To determine *N*-hydroxy-4-biphenylacetamide and *N*-hydroxy-2-fluorenylacetamide, incubation mixtures were extracted with ethyl acetate containing the authentic compounds (1.9 nmol and 4.5 nmol respectively) to bring the coefficients of variance to less than 10%.

Calculation of kinetic constants

Data obtained from the initial-velocity studies were plotted in a double-reciprocal form to check the linearity of the relationships. The best-fit values (means $\pm$ S.E.) of K_m and $V_{\max}$ were obtained by the method of least squares with the Taylor expansion [31].

Inactivation of PDHC by intrinsic pyruvate dehydrogenase kinase

PDHC was inactivated by the kinase (EC 2.7.1.99) in the presence of ATP- Mg^{2+} as described in [32], except that 2-mercaptoethanol was not added.

Assay of PDHC, E1, E2 and E3

PDHC-catalysed NADH formation activity (NADH activity) was assayed spectrophotometrically at 30 $^\circ\text{C}$ and pH 7.8 as described by Walsh et al. [33]. E1-catalysed DCPIP reduction activity (DCPIP activity) was assayed spectrophotometrically at 30 $^\circ\text{C}$ and pH 7 as described in [24]. E2 and E3 activities were assayed as in [34] and [35] respectively.

m-Chloro-*p*-aminophenol from *o*-chloronitrosobenzene

Isolation and identification

The isolation of *m*-chloro-*p*-aminophenol, as a metabolite, was performed on a preparative scale with 45 mM sodium pyruvate, 0.7 mM TPP, 10 mM MgCl_2 , 590 μ mol of *o*-chloronitrosobenzene [added as 700 μ l of a bis-(2-methoxyethyl)ether solution], and porcine heart mitochondria (105 mg of protein), in a total volume of 14 ml at 30 $^\circ\text{C}$ with shaking. After 2 h, the nitroso compound (590 μ mol) and sodium pyruvate (630 μ mol) were added as solids. Mitochondria (30 mg of protein) were also

added every hour. After 4 h, NaOH (0.8 g) and NaCl (4.5 g) were added to the reaction mixture. The alkaline solution was washed with isopropyl ether (3 $\times$ 10 ml). Conc. HCl (1.7 ml) was added to the aqueous layer and the pH was then adjusted with NH_4HCO_3 to 6.7, followed by extraction with isopropyl ether (3 $\times$ 10 ml). The combined extracts were evaporated under reduced pressure, and the residue was separated by silica-gel t.l.c. (Merck; article 5554). After development with ethyl acetate/benzene (1:2, v/v), *m*-chloro-*p*-aminophenol was eluted with isopropyl ether from the plate and subjected to m.s.: (EI, 70 eV) m/z 143 (145) (M^+ , base peak), 80, 78 and 52. A portion of the compound was converted into the diacetylated derivative: ^1H -n.m.r. ($\text{Me}_2\text{SO}-d_6$, Me_4Si) δ (p.p.m.): 2.08 (3H, s), 2.26 (3H, s), 7.10 (1H, dd, J 2.6 and 8.9 Hz, H_b), 7.35 (1H, d, J 2.6 Hz, H_c), 7.67 (1H, d, J 8.9 Hz, H_a) and 9.56 (1H, s, NH, exchangeable with $^2\text{H}_2\text{O}$); m.s. (EI, 70 eV) m/z : 227 (229) (M^+), 185 (187), 143 (145) (base peak) and 43. Values in parentheses are the m/z of ions containing the isotope ^{37}Cl . High-resolution m.s.: calc. for $\text{C}_{10}\text{H}_9\text{NO}_3\text{Cl}$ 227.0349. Found: 227.0370 (error 2.1 milli-mass unit). These spectral data are identical with those of the authentic compounds.

Requirements for the formation of *m*-chloro-*p*-aminophenol

The incubation mixture contained 100 mM Mops/KOH (pH 6.8), 10 mM MgCl_2 , 0.5 mM TPP, 15 mM sodium pyruvate and 1 mM *o*-chloronitrosobenzene. Incubation was started by adding PDHC (20 μ g of protein), heat-inactivated (100 $^\circ\text{C}$ for 15 s) PDHC, isolated E1 (9.5 μ g of protein) or water (non-enzymic) and performed at 30 $^\circ\text{C}$ for 15 min. Non-enzymic rearrangement of *N*-hydroxy-*o*-chloroaniline or its *N*-acetyl compound (each 1 mM) to *m*-chloro-*p*-aminophenol was examined at 30 $^\circ\text{C}$ for 30 min in 100 mM Mops/KOH (pH 6.8) containing 0.5 mM TPP, 10 mM MgCl_2 and 15 mM sodium pyruvate.

RESULTS

N-Hydroxy-*N*-arylacetamide formation by mammalian heart mitochondria

When NOB and pyruvate were incubated in the presence of porcine heart mitochondria under the conditions described in Table 1, *N*-OH-AA, as well as azoxybenzene, was formed. Since a detectable amount of *N*-OH-AA is not formed without pyruvate

Table 1 Requirements for *N*-OH-AA formation by porcine heart mitochondria

The complete incubation mixture consists of 5 mM NOB, 10 mM MgCl_2 , 0.5 mM TPP and 15 mM sodium pyruvate. Each incubation was initiated by an addition of porcine heart mitochondria (0.24 mg of protein) and carried out at 30 $^\circ\text{C}$ with shaking (60 strokes/min). In the experiment with TTPP (0.1 mM), TPP was omitted. Heated mitochondria (0.24 mg of protein) were prepared by heating in boiling water saturated with $(\text{NH}_4)_2\text{SO}_4$ for 15 s. The values are means $\pm$ S.D. ($n = 3$).

Incubation mixture	<i>N</i> -OH-AA activity (nmol/min per mg of protein)
Complete	9.6 $\pm$ 0.4
— Pyruvate	< 0.1
— Mg^{2+}	7.4 $\pm$ 0.2
— TPP	6.9 $\pm$ 0.3
— Mitochondria	< 0.1
+ TTPP	5.5 $\pm$ 0.3
Heated mitochondria system	< 0.1

Table 2 *N*-Hydroxy-*N*-arylacetamide formation activities of mammalian heart mitochondria

The amounts of *N*-hydroxy-*N*-arylacetamides formed were determined at pH 6.8 and 30 °C in an assay medium containing 0.5 mM TPP, 10 mM MgCl₂, 15 mM sodium pyruvate and 0.25 mM nitroso compound. For 2-nitrosofluorene and 4-nitrosobiphenyl, Triton X-100 [final concn. 0.2% (w/v)] was added as a solubilizer. Incubation was performed for 30 min with shaking (60 strokes/min). N-OH-FAA and N-OH-BAA are abbreviations for *N*-hydroxy-2-fluorenylacetamide and *N*-hydroxy-4-biphenylacetamide respectively. Values are means $\pm$ S.D. ($n = 3$).

Mitochondria	Activity (nmol/30 min per mg of protein)		
	N-OH-AA	N-OH-FAA	N-OH-BAA
Bovine	90 $\pm$ 4	3.6 $\pm$ 0.2	3.6 $\pm$ 0.3
Human	36 $\pm$ 3	1.4 $\pm$ 0.2	1.4 $\pm$ 0.2
Porcine	76 $\pm$ 4	3.0 $\pm$ 0.3	2.8 $\pm$ 0.2

Figure 1 Initial-velocity profiles for PDHC-catalysed formation of N-OH-AA from NOB and pyruvate

Initial velocities of N-OH-AA formation were measured as described in the Experimental section. Incubation was initiated by adding PDHC (13 μ g of protein). (a) NOB as a variable substrate at different fixed concentrations of pyruvate (0.03, 0.06, 0.12, and 0.3 mM); (b) pyruvate as a variable substrate at different fixed concentrations of NOB (0.1, 0.2, 0.4, and 2 mM); (c), replotting of the intercepts of (a); (d) replotting of the intercepts of (b):

$$\frac{V_{\max}}{v} = 1 + \frac{0.05}{[\text{Pyruvate}]} + \frac{0.25}{[\text{NOB}]} + \frac{0.00}{[\text{Pyruvate}][\text{NOB}]}$$

or with heated mitochondria, the formation of N-OH-AA is a pyruvate-dependent enzymic reaction. On the other hand, azoxybenzene formed without pyruvate or with heated mitochondria is a pyruvate-independent non-enzymic reaction product (results not shown), as reported for the boar-spermatozoon-catalysed N-OH-AA formation [3]. The activity was enhanced by both Mg²⁺ and TPP, and inhibited by TTPP, a specific inhibitor of TPP-dependent enzymes [36]. The optimum pH and activation energy of N-OH-AA formation were 6.8 and 98 $\pm$ 4 kJ/mol (results not shown) respectively. These values are identical with those obtained from the isolated porcine heart PDHC (as shown in Table 5 below). This, together with the above-mentioned requirements for porcine heart mitochondrial activity, indicates that it is most likely mediated by a mitochondrial enzyme, namely PDHC.

To investigate species differences and substrate specificities in the formation of *N*-hydroxy-*N*-arylacetamides, the activity was also determined in bovine and human mitochondria (Table 2). Also serving as substrates, were carcinogenic 2-nitrosofluorene

Figure 2 pH-dependence of the kinetic parameters of PDHC-catalysed N-OH-AA formation

Initial velocities of N-OH-AA formation were measured at 13 mM NOB (for measurement of K_m^{pyruvate} or 15 mM-pyruvate (for measurement of K_m^{NOB}). (a) pK_m^{NOB} (●) and pK_m^{pyruvate} (○) versus pH; (b) $\log(V_{\max}/K_m^{\text{NOB}})$ (●) and $\log(V_{\max}/K_m^{\text{pyruvate}})$ (○) versus pH. The breakpoint denotes a pK_a value of 6.3.

and 4-nitrosobiphenyl, to give the corresponding *N*-hydroxy-*N*-arylacetamides. Ratios of the amounts of *N*-hydroxy-*N*-arylacetamides formed, i.e. N-OH-AA/*N*-hydroxy-2-fluorenylacetamide/*N*-hydroxy-4-biphenylacetamide = 25:1:1, were observed in all mitochondria tested.

N-OH-AA formation by PDHC

Isolated porcine heart PDHC-catalysed N-OH-AA formation was a TPP-dependent reaction (K_m 1.02 $\pm$ 0.12 μ M) and the activity was competitively inhibited by TTPP (K_i 0.22 $\pm$ 0.03 μ M) (results not shown). As shown in Figure 1, double-reciprocal plots of the data obtained from initial-velocity studies support a Ping Pong mechanism. The rate equation is shown in the legend. The apparent $V_{\max}$ was 0.36 $\pm$ 0.05 μ mol/min per mg of protein. The K_m^{pyruvate} and K_m^{NOB} were 0.05 $\pm$ 0.01 mM and 0.25 $\pm$ 0.07 mM respectively. The K_m^{pyruvate} was identical with that reported in the overall NADH activity of porcine heart PDHC [32]. In the pH-dependence of N-OH-AA formation catalysed by isolated porcine heart PDHC (Figure 2), K_m^{pyruvate} was independent of pH over the range (5.2–7.4) tested. K_m^{NOB} , however, increased at lower pH, though near-constancy of the value was observed over the pH range 6.7–7.4 (Figure 2a). Since NOB does not have any dissociable groups over the pH range tested, the pH- pK_m^{NOB} and pH- $\log(V_{\max}/K_m^{\text{NOB}})$ (Figure 2b) profiles seem to be due to ionization of an active-site functional group of pK_a 6.3. The optimum pH of 6.8, as shown in pH- $\log(V_{\max}/K_m^{\text{pyruvate}})$ profile (Figure 2b), for N-OH-AA formation activity is identical with the value reported for E1 of porcine heart PDHC [37].

Identification of the enzyme responsible for N-OH-AA activity

From the above results, E1 seems to be the essential component enzyme for the reaction. Since the activity of E1 is regulated

Figure 3 Time-dependent inactivation of PDHC mediated by pyruvate dehydrogenase kinase in the presence of ATP-Mg^{2+}

To 2.5 ml of a solution of PDHC (4.4 units of NADH activity, 0.79 mg of protein) in 50 mM-potassium phosphate (pH 7) at 30 °C, 50 μl of a solution of 5 mM ATP and 10 mM MgCl_2 in phosphate buffer was added. The residual NADH formation ($\bullet, \circ$), DCPIP reduction ($\blacktriangle, \triangle$) and N-OH-AA formation ($\blacksquare, \square$) activities of PDHC were measured at appropriate time intervals in the presence (filled symbols) or absence (open symbols) of ATP-Mg^{2+} . Each activity, assayed in 50 mM-potassium phosphate, pH 7, is plotted as a percentage of the untreated control. The apparent first-order-rate constants (k_{app} ; means $\pm$ S.E.) of inactivation, determined by semilogarithmic plots, were as follows: for the NADH activity, $k_{\text{app}} = (7.2 \pm 0.4) \times 10^{-4} \text{ s}^{-1}$; for the DCPIP activity, $k_{\text{app}} = (6.5 \pm 0.4) \times 10^{-4} \text{ s}^{-1}$; and for the N-OH-AA activity, $k_{\text{app}} = (6.5 \pm 0.7) \times 10^{-4} \text{ s}^{-1}$.

Table 3 DCPIP reduction, monoacetylhydrolipoamide (AL) formation, lipoamide (LA) reduction and N-OH-AA formation activities of PDHC and its component enzymes

E1 (DCPIP activity), E2 (monoacetylhydrolipoamide formation activity), E3 [lipoamide (LA) reduction activity] and N-OH-AA activities of PDHC and its isolated component enzymes (E1, E2 and E3) were assayed as described in the Experimental section. Ratio means N-OH-AA activity/DCPIP activity.

Enzyme	Specific activity ($\mu\text{mol/min per mg of protein}$)				Ratio
	DCPIP	AL	LA	N-OH-AA	
PDHC	0.037	3.5	1.1	0.38	10
E1	0.076	0.0	0.1	0.72	9.5
E2	0.007	4.4	0.0	0.07	10
E3	< 0.001	0.0	16	< 0.01	—

Figure 4 Cornish-Bowden plots for N-OH-AA formation catalysed by PDHC (a) and the isolated component enzyme E1 (b)

Initial velocities, measured at various concentrations of NOB in the presence of 15 mM pyruvate (saturated level) as described in the Experimental section, are expressed as $\mu\text{mol/min per unit}$, in which 1 unit of E1 reduces 1 μmol of DCPIP/min under the conditions used [24]. (a) K_m^{NOB} and V_{max} values were $0.26 \pm 0.08 \text{ mM}$ and $9.7 \pm 0.5 \mu\text{mol/min per unit}$ respectively. (b) K_m^{NOB} and V_{max} values were $0.34 \pm 0.07 \text{ mM}$ and $9.2 \pm 0.4 \mu\text{mol/min per unit}$ respectively. The K_m values agreed with those obtained in Figure 1(d).

through phosphorylation–dephosphorylation catalysed by pyruvate dehydrogenase kinase/phosphatase [4], a time course for the loss of enzymic activities of the PDHC was monitored for the

Table 4 Substrate specificities for α -oxo acids in DCPIP reduction and N-hydroxy-N-phenylacetylamine formation activities of PDHC

DCPIP reduction and N-hydroxy-N-phenylacetylamine (N-OH-PA) formation activities of PDHC were assayed at the concentration of 15 mM- α -oxo acid in 100 mM Mops/KOH, pH 6.8. Activities are expressed relative to pyruvate ($R = \text{CH}_3$), arbitrarily taken as 100.

α -Oxo acid ($R\text{-CO-COOH}$)		Relative activity	
R	E_s	DCPIP	N-OH-PA
CH_3	0	100	100
CH_3CH_2	−0.07	55	82
$\text{CH}_3\text{CH}_2\text{CH}_2$	−0.36	32	0.0
$(\text{CH}_3)_2\text{CH}$	−0.47	24	0.0
$\text{CH}_3\text{CH}_2(\text{CH}_3)\text{CH}$	−1.13	7	0.0
$(\text{CH}_3)_2\text{CHCH}_2$	−0.93	3	0.0

kinase-mediated inactivation of PDHC. As shown in Figure 3, in the presence of ATP-Mg^{2+} , the N-OH-AA activity as well as the DCPIP and NADH activities decreased in a pseudo-first-order manner for up to about 55 % inhibition. No significant differences were observed among the three first-order rate constants (see the legend to Figure 3). This result indicates that the N-OH-AA activity may be attributed to the active (non-phosphorylated) form of E1.

To confirm further that E1 is the enzyme responsible for the N-OH-AA activity of PDHC, N-OH-AA activities of PDHC and its major component enzymes (E1, E2, and E3) were assayed (Table 3). E1 showed the highest N-OH-AA activity, whereas the activity was not detected in E3. From the ratio between the N-OH-AA and DCPIP activities, the N-OH-AA formation is shown to be mediated by E1 alone. A weak N-OH-AA activity detected in E2 seems to be due to contamination by E1, because the value of the ratio agreed with those obtained from E1. As observed in the Cornish–Bowden plots [38] (Figure 4), there are no significant differences between the values of the kinetic parameters obtained for N-OH-AA activities of PDHC and its isolated component, E1. Furthermore, the values for activation energy ($98 \pm 10 \text{ kJ/mol}$) and the optimum pH (6.8) of E1-catalysed N-OH-AA formation were identical with those obtained with PDHC (see Table 5 below).

N-Hydroxy-N-phenylacetylamine from NOB and α -oxo acids

To investigate the specificity of α -oxo acids as substrates in the formation of the corresponding N-hydroxy-N-phenylacetylamine, the reactivity of NOB with six kinds of α -oxo acids, including pyruvate, was examined. As shown in Table 4, pyruvate and α -oxobutyrate were found to be effective substrates, whereas formation of N-hydroxy-N-phenylacetylamine derived from the other α -oxo acids could not be detected. In the DCPIP activity, however, all α -oxo acids tested served as substrates, though there was high specificity for pyruvate and α -oxobutyrate. Excellent correlation of log(relative DCPIP activity) versus the σ^* (polar effect) and E_s (steric effect) parameters of the R group of α -oxo acids was observed using the Taft equation [39]: the correlation equation is as follows:

$$\begin{aligned} \log(\text{Relative DCPIP activity}) \\ = 1.28E_s - 0.98\sigma^* + 1.84 \\ (1.03) \quad (0.13) \end{aligned}$$

$$r = 0.939, n = 6, F = 11.3 (P < 0.05)$$

Figure 5 Correlation of log(relative DCPIP activity) of PDHC with Taft's E_s constants of α -oxo acids

DCPIP activities were expressed relative to pyruvate ($R = \text{CH}_3$), arbitrarily taken as 100 (see Table 4). The correlation equation obtained was as follows:

$$\log(\text{relative DCPIP activity}) = (1.16 \pm 0.14)E_s + (1.90 \pm 0.14)$$

Table 5 Kinetic parameters of the PDHC-catalysed formation of *N*-hydroxy-*N*-arylacetamides

Values of K_m (mM) for the nitroso compounds were measured in the presence of 15 mM sodium pyruvate at pH 6.8. $V_{\max}$ values are expressed as nmol/min per unit, in which 1 unit of PDHC forms 1 μmol of NADH/min under the conditions described for NADH activity [33]. Activation energy (E_a ; means $\pm$ S.E.) was obtained by Arrhenius plots (at 24–36 °C). For *o*-chloro-NOB (*o*-Cl-NOB), values in parentheses are for the formation of *m*-chloro-*p*-aminophenol. For 4-nitrosobiphenyl (NOBP) and 2-nitrosofluorene (NOF), Triton X-100 (final concn. 0.2%) was added to the incubation mixture as a solubilizer.

Nitroso compound	Optimum pH	K_m (mM)	$V_{\max}$ (nmol/min per unit)	E_a (kJ/mol)
NOB	6.8	0.31 ± 0.06	65 ± 4	96 ± 8
<i>o</i> -Cl-NOB	6.8	0.28 ± 0.06	79 ± 8	94 ± 8
	(6.8)	(0.24 ± 0.03)	(29 ± 3)	(88 ± 8)
<i>m</i> -Cl-NOB	6.8	0.24 ± 0.02	104 ± 4	79 ± 4
<i>p</i> -Cl-NOB	6.8	0.30 ± 0.04	71 ± 4	100 ± 8
<i>p</i> -Me-NOB	—	0.30 ± 0.03	31 ± 1	—
<i>p</i> -OEt-NOB	—	0.27 ± 0.02	19 ± 1	—
NOBP	—	0.30 ± 0.02	3.5 ± 0.3	—
NOF	—	0.35 ± 0.04	2.7 ± 0.3	—

From the standard partial regression coefficients shown in parentheses in the above equation, E_s is found to be more important than σ^* . This result is also supported by the good correlation between log(relative DCPIP activity) with the E_s parameter alone ($r = 0.935$, $n = 6$, $P < 0.01$; see Figure 5). The positive value (1.28) of δ , a proportionality constant giving the susceptibility of a reaction to steric effects, indicates that the activity of E1-catalysed oxidative decarboxylation of α -oxo acids decreases with the increase in steric hindrance of the R group of α -oxo acids.

Since 2-(1-hydroxyethyl)-TPP, an isolated product in the enzymic decarboxylation of pyruvate [8], is reportedly degraded by a pyruvate-decarboxylating component of pigeon breast muscle PDHC [40], N-OH-AA formation from NOB and 2-(1-hydroxyethyl)-TPP was examined. When 2-(1-hydroxyethyl)-TPP was incubated with NOB in 100 mM Mops/KOH, pH 6.8, at 30 °C, no detectable N-OH-AA was formed, either in the presence or in the absence of PDHC. This result indicates that 2-(1-hydroxyethyl)-TPP is not a direct substrate reacting with NOB.

Figure 6 Correlation of $\log(V_{\max}/K_m)$ for nitroso aromatic compounds in the formation of *N*-hydroxy-*N*-arylacetamides versus Hammett's σ^* constants

The correlation equation ($r = 0.975$, $n = 5$, $P < 0.01$), obtained except for NOBP, was as follows:

$$\log(V_{\max}/K_m) = (0.69 \pm 0.09)\sigma^* + (2.32 \pm 0.04)$$

Abbreviations: OEt, ethoxy; Me, methyl.

Effects of substituents of NOB on the activity

Several nitroso aromatic compounds, including carcinogenic ones, were studied for their reactivities in the PDHC-catalysed formation of *N*-hydroxy-*N*-arylacetamides to reveal the mechanistic aspects of the reaction. As summarized in Table 5, values of K_m for the nitroso compounds were not significantly different. $V_{\max}$ values, however, were affected by both the ring substituents and their position. From the positive ρ value in the Hammett plot [41] versus σ^* (Figure 6), it is suggested that nitroso aromatic compounds serve as electrophiles. The extremely lowered reactivity of 4-nitrosobiphenyl suggests that an additional factor, e.g., a steric effect, should be considered. By the application of Hammett (σ^*)-Taft (E_s) equation to NOB and *p*-substituted (chloro-, methyl- and phenyl-) nitrosobenzenes, a good correlation of $\log(V_{\max}/K_m)$ versus E_s values is obtained. The equation is as follows:

$$\log(V_{\max}/K_m) = 0.33E_s + 0.56\sigma^* + 2.08$$

(0.88) (0.17)

$$r = 0.970, n = 4, F = 8.00 (P < 0.05)$$

From the standard partial regression coefficients shown in parentheses in the above equation, the steric effect was found to be more important than the electronic effect on the catalytic efficiency of PDHC.

Using *o*-chloronitrosobenzene, formation of *m*-chloro-*p*-aminophenol and *N*-hydroxy-*o*-chloroaniline was concomitant with the formation of *N*-hydroxy-*o*-chloroacetanilide and was shown to be due to a pyruvate-dependent enzymic reaction catalysed by PDHC as well as the isolated E1 and porcine heart mitochondria (results not shown). Non-enzymic rearrangement of *N*-hydroxy-*o*-chloroaniline or its *N*-acetyl compound to *m*-chloro-*p*-aminophenol was not observed. The values of optimum pH and the activation energy of the formation of *m*-chloro-*p*-aminophenol and K_m for *o*-chloronitrosobenzene in the reaction were identical with those obtained for the formation of *N*-hydroxy-*o*-chloroacetanilide (Table 5), indicating that both the metabolites can be formed from a common intermediate.

The oxidative decarboxylation step catalysed by E1 is reported to be rate-limiting in the overall reaction [42]. The activation energy of the overall NADH formation of the isolated porcine

heart PDHC, in 100 mM Mops/KOH, pH 6.8, was 47 ± 2 kJ/mol (results not shown), being smaller than the values of activation energy obtained in the PDHC-catalysed formation of *N*-hydroxy-*N*-arylacetamides (listed in Table 5).

DISCUSSION

Formation of *N*-hydroxy-*N*-arylacetamides from nitroso aromatic compounds and pyruvate has been observed in bovine, human and porcine heart mitochondria. In porcine heart mitochondrion-catalysed *N*-OH-AA formation (Table 1) there are characteristics identical with those of the reaction catalysed by mammalian spermatozoa [3]; the activity of *N*-OH-AA formation was enhanced by TPP and inhibited by TTPP. The optimum pH and activation energy of the mitochondrion-catalysed reaction are the same as those of the isolated porcine heart PDHC-catalysed reaction. These results indicate that the mitochondrion-catalysed formation of *N*-hydroxy-*N*-arylacetamides is mediated by PDHC.

N-OH-AA formation catalysed by isolated porcine heart PDHC is shown to proceed through a Ping Pong mechanism (Figure 1) which is also characteristic of the normal PDHC reaction [43]. Since the isolated component enzyme E1 shows the full *N*-OH-AA activity (Table 3 and Figure 4), E1 functions without any conformational changes affecting the activity due to it being detached from the other component enzymes (E2 and E3) of PDHC. The normal reaction catalysed by E1 reportedly proceeds in two stages, in which pyruvate is decarboxylated to form 2-(1-hydroxyethylidene)-TPP, followed by reductive

acetylation of the lipoyl residue covalently bound to E2 [9]. From the value of K_m^{pyruvate} in the PDHC-catalysed formation of *N*-OH-AA, the first step of pyruvate decarboxylation is thought to proceed in the same way as PDHC in the normal catalytic reaction. At the next step, however, nitroso aromatic compounds, in place of the E2-bound lipoyl moiety, are thought to occupy a transacetylation site of E1 and then to be reductively acetylated. As shown in Figure 2, variation in the kinetic parameters as a function of pH can be presumed to be due to enzymic ionization affecting the catalytic efficiency. The pH profiles for pK_m^{NOB} and $\log(V_{\text{max}}/K_m^{\text{NOB}})$ are dependent on the ionization behaviour of the enzymic group with pK_a 6.3. This probably participates in the second step, i.e. reductive transacetylation of NOB. The group may be attributed to a histidine imidazole residue, which reportedly plays an essential role in the catalytic function of E1 [44].

A proposed mechanism for the formation of *N*-hydroxy-*N*-arylacetamides catalysed by PDHC is shown in Scheme 1. In the first step of the decarboxylation reaction there were large differences in the substrate efficiencies of six α -oxo acids, as observed in the DCPIP activity (Table 4). This is attributed to the steric effect of the R group (see Figure 5) on the active site of E1, as indicated by Khailova et al. [44]. A high steric limitation to pyruvate and α -oxobutyrate, in the formation of *N*-hydroxy-*N*-phenylacetamides (Table 4), indicates that the bulkiness of the R group also affects the catalytic efficiency in the second step, i.e. a nucleophilic addition of 2-(1-hydroxyalkylidene)-TPP to nitroso compounds at the active site of E1. Judging from the activation energies, this nucleophilic reaction is rate-limiting. The fact that an electron-withdrawing substituent made the nitroso compounds susceptible to the reductive acetylation (Table 5 and Figure 6), as reported in the transketolase-catalysed reaction [27], supports the above-mentioned mechanism in which nitroso compounds serve as electrophiles. As for the steric effect of substituents, a bulky *p*-substituent lowers the catalytic efficiency, owing to the steric limitation of the active site in E1. 2-Chloronitrosobenzene, having an *o*-chloro substituent, however, did show a comparable activity with that of NOB (Table 5). This indicates that the steric effect of the *o*-chlorine substituent affects neither the affinity of the nitroso compound for the active site of E1 nor the catalytic efficiency of the reductive transacetylation. *o*-Substituents have been reported to cause steric acceleration in the reduction of nitro aromatic compounds [45]. In addition, *o*-substituted nitroso aromatic compounds reportedly possess higher oxidation-reduction potentials than NOB [13]. Therefore the unusual observation that the *o*-chloro substituent does not affect the catalytic efficiency might be explained by thinking that the steric effect of the *o*-chloro substituent is offset by raising the oxidation-reduction potential. Using *o*-chloronitrosobenzene, *N*-hydroxy-*o*-chloroaniline and *m*-chloro-*p*-aminophenol (a Bamberger rearrangement product) were detected with concomitant formation of *N*-hydroxy-*o*-chloroacetanilide. These compounds are thought to be derived from *N*-acetoxy-*o*-chloroaniline, a labile compound susceptible to N–O-bond cleavage. The *O*-acetyl compound seems to be immediately hydrolysed to the *N*-hydroxylamino derivative and to rearrange to *m*-chloro-*p*-aminophenol, because steric hindrance of the *o*-substituent has been reported to facilitate the Bamberger rearrangement of *N*-hydroxyanilines by the elongation of the N–O bond, owing to the relaxation of steric crowding [46]. The reaction pathways shown in Scheme 1 are assumed to account for this finding. The speculative formation of the *O*-acetyl compound may be explained by a rearrangement (path b) of (B) formed from (A). The formation of (B) from (A) via an intramolecular substitution at the carbinol carbon is a pathway to diminish the steric hindrance

Scheme 1 A proposed mechanism of E1-catalysed *N*-OH-AA formation

of the *o*-chloro substituent ($X = \text{Cl}$) in (A). Since an oxaziridine derivative, such as (B), has been reported to be a tautomer of the corresponding *N*-hydroxy-*N*-arylacylamide [47], the pathway by which both *N*- and *O*-acyl compounds are formed via (B) seems reasonable. Thermodynamically favourable *N*-hydroxy-*N*-arylacylamides can be formed from (B) by path a (Scheme 1). In the *o*-chloro derivative ($X = \text{Cl}$), however, path b yielding a less hindered *O*-acyl compound than the corresponding *N*-acyl compound, can proceed competitively with path a.

It has been reported that 2-acetyl-TPP is a chemically competent intermediate at the active site of E1 and is present in the steady state of the overall reaction [11,48]. Also, 2-acylthiazolium salts, modelled upon the biologically important 2-acylthiamine derivatives, react specifically with the oxygen atom of *N*-hydroxyaniline to give *O*-tetrahedral intermediates, which collapse to the *O*-acyl derivatives under mildly basic conditions [49]. From these findings, an intermolecular redox reaction between nitroso compounds and 2-(1-hydroxyalkylidene)-TPP, yielding the corresponding *N*-hydroxylamino compounds and 2-acyl-TPP may be another possible pathway for the formation of the *N*- and *O*-acyl derivatives. However, the following transacylation reaction is thought to be impossible from the perspective of the Law of Mass Action, though the possibility is not excluded completely, provided that the *N*-hydroxylamino compounds are tightly associated with the active site of E1 and are efficiently transacylated. Since all nitroso aromatic compounds tested, except 2-chloronitrosobenzene, did not appear to yield corresponding *O*-acyl compounds, it is suggested that the nucleophilic pathway (one step) for the formation of (A) shown in Scheme 1 is more reasonable compared with the above-mentioned pathway (two steps) involving the 2-acyl-TPP intermediate.

N-Hydroxy-*N*-arylacetamides are thought to be proximate carcinogens which are further biotransformed to reactive species capable of covalently binding to nuclear DNA [1,50]. Mitochondrial DNA is also reported to be a sensitive target for some chemical carcinogens, including *N*-substituted aromatic compounds [51]. Therefore the biotransformation of nitroso aromatic compounds into the corresponding proximate carcinogenic *N*-hydroxy-*N*-arylacetamides could be responsible for carcinogenicity and/or necrosis in tissues which are low or lacking in cytochrome-*P*-450-catalysed *N*-oxidation activity.

PDHC catalyses oxidative decarboxylation of pyruvate, resulting in the production of acetyl-CoA and NADH [4], and hence plays central roles in cellular energy metabolism. Therefore, the E1-catalysed formation of *N*-hydroxy-*N*-arylacetamides implies inhibition of this function of PDHC. This may result in a decreased intracellular concentration of ATP, though PDHC is not the only enzyme participating in cellular energy metabolism. Since the activity of E1 in the formation of *N*-hydroxy-*N*-arylacetamides is regulated by ATP (Figure 3), under such a shortage of ATP, E1 is thought to continue forming *N*-hydroxy-*N*-arylacetamides because the intrinsic ATP-dependent pyruvate dehydrogenase kinase would be unable to function. Accordingly, the PDHC-catalysed formation of *N*-hydroxy-*N*-arylacetamides may exert toxic effects on all cells possessing mitochondria, not only by causing damage to both nuclear and mitochondrial DNA, but also by impairing the energy and material metabolism of the cells.

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